SCREENING FOR MICROALBUMINURIA IN GHANAIAN DIABETIC PATIENTS: ALBUMIN BLUE 580 FLUORESCENCE PROBE EVALUATED.

A THESIS SUBMITTED

BY

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DECLARATION.

I CARRIED OUT THE EXPERIMENTAL WORK DESCRIBED IN THIS PROJECT AND ALL REFERENCES CITED IN THS WORK HAVE BEEN FULLY ACKNOWLEDGED.

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DEDICATION.

To my 3 children: Harold, Terry, and Wendy and all Diabetic patients in Ghana.

"……..and gladly would be learned and gladly teche……."

(From Canterbury-Chaucer 1342-1400)

ABSTRACT

The Albumin Blue 580 fluorescent dye was evaluated for the measurement of urine albumin content. The calibration of the assay was linear up to 150.0mg/L with a minimum detection limit of 1.4mg/L. The intraassay CV was 4.3%(14.5mg/L), 3.7%(50.2mg/L) and 1.4%(100.0mg/L), interassay CV was 2.7%, 2.6% and 3.7% for the respective urine albumin concentrations. Recovery of added albumin to urine was 95-112%, reproducibility for 9 replicate analysis of 3 urine samples was 8.2%(6.0mg/L), 4.6%(62.4mg/L) and 4.9%(130.2mg/L). Storage at -20°C and -80°C beyond 14 days may misclassify borderline cases of normoalbuminuria and microalbuminuria.

In the non-communicable disease survey aliquots of 2hour albustix negative urine were obtained from 47 newly diagnosed diabetic subjects, 52 non-diabetic, non-hypertensive subjects as a control group. The age, systolic blood pressure, diastolic blood pressure and AER were significantly lower in the control subjects ($p<0.05$ (38.7 ± 8.6 vrs 47.3 ± 9.0 yrs;), (119.1 ± 10.1 vrs 137.3 ± 22 mmHg); (77.7 ± 7.5 vrs 88.6 ± 13.0 mmHg) and (11.6 ± 1.9 vrs 34.6 ± 2.1μg/min) respectively. 40 (85.1%) and 7 (13%) of diabetic and non-diabetic subjects respectively had AER ≥ 20.0μg/min. There were 7 proposals of UAC cut off values in predicting overnight AER of ≥ 15.0 μg/min and ≥ 20.0μg/min. Proposal VI with UAC of 25.0mg/L in predicting overnight AER ≥ 20.0μg/min had a diagnostic sensitivity of 100% for the newly diagnosed diabetic subjects and specificity of 87% in the non-diabetic subjects for the absence of microalbuminuria and a positive predictive value of 97.5%. At a lower UAC of 10.0mg/L sensitivity was 100% for both groups but the specificity reduced to 32% and 16.7% for diabetic and non- diabetic subjects respectively. The prevalence of elevated N-AER in 212 diabetic patients providing a single 12hour overnight urine collection was 37.7%. The diagnostic accuracy , Likelihood ratio of a positive test result within this population and the post-test probability were 92%, 7.7 and 82.5% respectively , making the AB 580 assay method a useful and a reliable test for the screening and diagnosis of early diabetic kidney disease in Ghanaian diabetic subjects and for evaluation of risk factors for the progression of intermittent to persistent microalbuminuria.

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ABBREVIATIONS

AB 580	Albumin Blue 580 dye
ACE	Angiotensin converting enzyme.
ACEI	Angiotensin converting enzyme inhibitor.
ACR	Albumin creatinine ratio.
ADA	American Diabetes Association.
AGE	Advanced glycated end products.
BP	Blood pressure.
BDH	British Drug House.
CBB	Coomassie brilliant blue.
CEIA	Competitive-Enzyme Immunoassay.
CI	Confidence Interval.
DCCT	Diabetes Complications and Control Trials.
DNA	Deoxyribonucleic acid.
EDTA	Ethylenediamine tetraacetic acid.
ELISA	Enzyme-linked immunosorbent assay.
ESRD	End-stage renal disease
FIA	Fluorescent Immunoassay.
FMU	First morning urine.
FN	False negative.
FP	False positive.
GCW	Glomerular capillary wall
GBM	Glomerular basement membrane.
GDM	Gestational diabetes mellitus.
GFR	Glomerular filtration rate.

GSH Glutathione (reduced form).

GSSG Glutathione (oxidized form).

HLA Human leucocytic antigen.

HSPG Heparan sulfate proteoglycan.

IE Immuno-electrophoresis.

IgG Gamma Immunoglobulin.

IN Immuno-nephelometry.

IT Immunoturbidimetry.

IDDM Insulin dependent diabetes mellitus.

JNC –V Joint National Committee 5th Report.

LDL Low density lipoprotein.

MAU Microalbuminuria

MOPS 3-N-Morpholinosulphonic acid.

N-AER Overnight albumin excretion rate.

NAD^+ Nicotinamide adenosine dinucleotide (oxidized).

NADH Nicotinamide adenosine dinucleotide (reduced).

$NADP^+$ Nicotinamide adenosine dinucleotide phosphate (oxidized).

NADPH Nicotinamide adenosine dinucleotide phosphate (reduced).

NAG N-acetyl-β-D-glucosaminidase.

NCDS Non-communicable disease survey.

NDDG National Diabetes Data Group.

NIDDM Non-insulin dependent diabetes mellitus.

NPV Negative predictive value.

PPV Positive predictive value.

Pr Prevalence.

RBP Retinol binding protein.

RNA	Ribonucleic acid.
ROC	Receiver operator characteristics.
SDS	Sodium dodecyl sulfate.
Sens.	Diagnostic sensitivity.
Spec.	Diagnostic specificity.
TER_{alb}	Trans-capillary escape rate of albumin.
TGF	Transforming growth factor.
TN	True negative.
TP	True positive.
UAC	Urine albumin concentration.
UK	United Kingdom.
USA	United States of America.
UTI	Urinary tract infection.
WHO	World Health Organization.

CHAPTER ONE

1.0: INTRODUCTION AND LITERATURE REVIEW

1.1: INTRODUCTION:

Diabetes mellitus is a complex metabolic disorder characterized by hyperglycemia resulting from defects of insulin secretion, insulin action or both. It is associated with derangement of normal metabolism of carbohydrate, lipids and proteins. The chronic hyperglycemia in diabetes is associated with long-term damage, dysfunction and failure of several organs in the body. The extent of tissue damage and complications of diabetes are influenced by the severity of the diabetic state and the extent of diabetic control *(Expert Committee of ADA, 1998).* There are several pathogenic processes involved in the development of diabetes ranging from autoimmune destruction of the β-islet cells of the pancreas with consequent insulin lack to abnormalities that result in resistance to insulin action.

In 1985, the WHO approved a classification and criteria for the diagnosis of diabetes. Two major forms were recognized, they were termed insulin dependent diabetes mellitus *(IDDM, Type 1 diabetes)* and non-insulin dependent diabetes mellitus *(NIDDM, Type 2 diabetes).* This classification included etiological and other clinical heterogeneous group of disorders that have hyperglycemia in common. In 1995, the American Diabetes Association (ADA) sponsored an Expert Committee to review the previous classification and diagnostic criteria for diabetes. The Expert Committee recommended that the terms IDDM and NIDDM should be eliminated as they are confusing and result in classifying patients based on treatment rather than etiology. They retained the classification of Type 1 and Type 2 diabetes with Arabic numeral rather Roman numerals (*Expert Committee of ADA, 1998).*

Type 1 diabetes encompasses the vast majority of cases that are primarily due to pancreatic β-islet cell destruction and prone to keto-acidosis. It includes cases ascribable to an autoimmune process and others where the etiology is not known, but excludes non-autoimmune causes of β-cell destruction e.g. cystic fibrosis. Type 2 diabetes includes cases ranging from a predominantly insulin resistance with relative insulin deficiency to predominantly insulin secretion with insulin resistance. Another major class of diabetes is gestational diabetes mellitus (GDM), with any degree of glucose intolerance with onset or first recognized during pregnancy.

The clinical manifestations of marked hyperglycemia include polyuria, polydipsia, weight reduction, sometimes hyperphagia and visual impairment. In some cases there are no symptoms. This is common in Type 2 diabetes where hyperglycemia may be present for sometime before it manifests clinically. The diagnostic criteria for diabetes mellitus have been modified from those recommended by *National Diabetes Data Group NDDG (1976) and WHO (1985)* by the *Expert Committee of ADA (1998).* They are as follows:

1. Symptoms of diabetes and a casual plasma glucose concentration ≥ 11.1 mmol/L;
2. A fasting plasma glucose concentration (FPG) ≥ 7.0 mmol/L
3. A 2-hour post-glucose (2hPG) ≥ 11.1 mmol/L during an Oral Glucose Tolerance Test (OGTT),

where the casual plasma glucose is defined as plasma glucose level at anytime of the day without regard to the last mealtime. Fasting is defined as no caloric intake for at least 8 hours and the OGTT as recommended by WHO using 75g of anhydrous glucose dissolved in water. However, in the absence of unequivocal hyperglycemia with acute metabolic de-compensation, these criteria must be confirmed by repeat testing on a different day, but the OGTT is not recommended for routine clinical use *(Expert Committee of ADA, 1998).*

Diabetes mellitus is a major cause of morbidity and premature deaths in the US, UK and Denmark (*Hirsch, 1996)*. There are several complications associated with the disease, both short and long term. In the short term there is growth impairment, increase susceptibility to infections, some life threatening conditions like keto-acidosis or the non-ketotic hyper-osmolar syndrome. The long-term complications are retinopathy with potential visual loss, nephropathy leading to end-stage renal disease (ESRD), peripheral neuropathy with risks of foot ulcers and increased frequency of limb amputations, autonomic neuropathy affecting the gastrointestinal, genito-urinal and cardiovascular systems and finally sexual dysfunction.

Diabetes is the leading cause of ESRD in UK and USA, accounting for 30% of all ESRD entering chronic dialysis programs. The number of diabetic subjects entering dialysis programs is increasing, in part due to the growing incidence of diabetes *(ADA-Consensus Statement, 1994; Hirsch; 1996).* The majority of patients entering dialysis programs have Type 2 diabetes although a substantial portion of ESRD cases have Type 1 diabetes. This is due to the fact that nephropathy complicates Type 1 more frequently than Type 2 diabetes. However as there are many more cases of Type 2 diabetes in the general population, a lot more cases of Type 2 enter ESRD programs.

The evidence for overt or clinical diabetic nephropathy is the presence of proteinuria as detected by dipsticks. Prior to the detection of overt proteinuria there is evidence of intra-glomerular hypertension, glomerular hyper-filtration and an increase in albumin excretion rate (AER). Without intervention, microalbuminuria progresses to clinical nephropathy. When clinical nephropathy develops, systemic hypertension occurs over baseline blood pressure and if the resulting microalbuminuria is not treated, nephritic syndrome occurs resulting in the development of ESRD *(Hirsch, 1996).* Prevention of diabetic nephropathy is very important because patients with diabetes

have 50% greater risk of morbidity and mortality on dialysis than do patients without diabetes *(Consensus Statement, 1994).*

In Ghana, chronic or maintenance dialysis is available at the National Cardiothoracic Centre of the Korle Bu Teaching Hospital, where it costs approximately ¢60 million per person per year *(Amoah).* This is beyond the means of the majority of Ghanaians. The early detection and treatment of sub-clinical nephropathy or microalbuminuria will therefore be cost effective. Diabetic subjects should initially be screened for macroalbuminuria. Those who test negative are to be screened for microalbuminuria. Subjects who are found to have microalbuminuria are to be offered treatment.

The assay of micro albumin will require a sensitive, specific, safe, cheap and robust method. Current methods available specifically for the determination of micro albumin are expensive. They have a long turnaround time. Though they are sensitive and specific, isotopic assays are potentially hazardous. The Albumin Blue 580 fluorescence dye-binding assay is non-immunologic, non-isotopic, robust, cheap, safe, sensitive, and specific that can be used on any conventional spectroflourometer.

This study was undertaken to assess the various analytical variables of AB 580 assay, and to employ it in the screening of microalbuminuria in a community and clinic sample of diabetic subjects.

1.2: LITERATURE REVIEW

1.2.1: Diabetic Nephropathy

Long standing diabetes mellitus may be complicated by micro- and macro vascular disease. The vascular complication of clinical importance is diabetic angiopathy. In diabetic micro-angiopathy there are disruptions of the normal function of capillary bed. The feature of diabetic microangiopathy is significant thickening and or sometimes duplication of the micro vascular basement membrane with

chronic progression as a function of time. Paradoxically, the thickening of the micro vascular basement membrane is accompanied by loss of function allowing charged macromolecules e.g. (albumin, Mr. 68kDa) that are normally retained within the circulation to pass across the matrix. This loss of function produces systemic effects in all organs of the body, but more importantly the glomeruli in the kidneys and retinal blood vessels in the eyes of diabetic patients. (*Gosling, 1995a; Rodico et al, 1998*).

The presence of persistent proteinuria detectable clinically by the conventional laboratory reagent strip (Albustix) in the diabetic subjects heralds the onset of diabetic nephropathy. Diabetic nephropathy is a clinical syndrome characterized by (i) persistent proteinuria > 500mg/24hr or albuminuria > 300mg/24hr, (ii) elevation of blood pressure and (iii) declining glomerular filtration rate (GFR) (*Mathieson, 1993).* Diabetic nephropathy is a common cause of morbidity and mortality in both types of diabetes mellitus (*Wang et al, 1996).* It is invariably associated with longer duration of diabetes in Type 1 subjects, systemic hypertension, elevated serum creatinine and retinopathy *(Viberti and Keen, 1984;Rowe et al, 1990).* At this stage the management of hypertension, glycaemia and possibly dietary protein restriction can at best delay its progression towards ESRD. Patients with ESRD require dialysis or kidney transplantation. These modalities of treatment are rather expensive and are associated with a higher mortality in the diabetic subjects than in non-diabetic subjects. Diabetic nephropathy is a leading cause of ESRD in the U.S. and U.K, accounting for 30-40% of all cases presenting in kidney transplantation programs *(Friedman, 1982).* It is estimated that managing diabetic nephropathy will cost over $3 billion in the next decade. Worldwide, it is currently estimated that 100,000 cases are currently receiving kidney maintenance therapy *(Steffes, 1997; Alzaid, 1996)* and that this figure is likely to grow as more elderly patients are being admitted into dialysis programs. The onset of nephropathy is one of progressive and relentless decline in kidney function. The process is usually irreversible and often refractory to treatment. It also renders the diabetic patient vulnerable to atherosclerotic disease and its attendant morbidity and mortality. The most

effective means to prevent diabetic nephropathy is directed towards its early detection and treatment at its very onset where the response to therapy is best *(Alzaid, 1996).*

1.2.2: MICROALBUMINURIA:

1.2.2.1 Definitions:

Microalbuminuria (MAU) is defined as an increased urinary excretion of albumin above the normal range for healthy non-diabetic subjects and not detectable by the Albustix tests *(Viberti and Keen, 1984)*. The problem with this definition of MAU is its description relative to the Albustix, which by itself is non-specific, semi-quantitative and may misclassify patients whose urine are dilute or concentrated. For these reasons, microalbuminuria has been defined in various ways from different studies:

(i) albuminuria that is greater than normal – (2.5 – 26mg/24hr) but not detectable by conventional dipstick (< 250mg/24hr) *(Townsend, 1990).*

(ii) urine albumin concentration of 30-140μg/ml *(Viberti et al, 1982).*

(iii) urine AER of 15-150μg/minute or 30-300mg/24hr*(Viberti et al, 1982)*; 20-200μg/min (*Mogensen et al,1995*).

(iv) urine albumin concentration to creatinine concentration ratio (ACR) of 2.5 – 25mg/mmol or 30-300 mg/g *(Mogensen et al, 1995)* or 3-30mg/mmol.

Table 1.0 shows a recent classification of albumin excretion by *Ruggenti and Remuzzi (1998).* In this scheme non-timed urine is defined as first morning urine (FMU) or random void urine and timed urine defined as overnight and 24-hour collection with corresponding figures of microalbuminuria.

Table1.1: Classification of Microalbuminuria by Ruggentti and Remuzzi. (1998)

	NON-TIMED URINE COLLECTION*		TIMED URINE COLLECTION	
	Unadjusted	***Adjusted for creatinine conc.***	***Overnight***	***24hr***
Normoalbuminuria	< 20μg/ml	< 30mg/g	< 20 μg/min	< 30mg/24hr
Microalbuminuria	20 - 200μg/ml	30 – 300mg/g	20 - 200μg/min	30 – 300mg/24hr
Macroalbuminuria	> 200 μg/ml	> 300mg/g	> 200μg/min	> 300mg/24hr

** In the absence of any urine abnormality and urinary tract infection*

The hallmark of overt diabetic nephropathy is persistent proteinuria ≥ 500mg/24hr without clinical or laboratory evidence of other forms of kidney disease or kidney tract disease other than glomerulosclerosis. Persistent proteinuria of ≥ 500mg/hr approximates to ≥ 300mg/24hr of urinary albumin excretion. These definitions rely on excluding non-diabetic causes of albuminuria. In addition, proteinuria must be noted in at least 2 out of 3 consecutive urinary collections within 3-6 months. In diabetic patients with proteinuria it is therefore advisable to include at least 3 urinary collections for assessment of proteinuria.

The consensus definition of microalbuminuria is AER within 20-200 μg/min in at least 2 out of 3 consecutive, non-ketotic, sterile urine samples with normal microscopy within a 6-month period *(Consensus conference, 1994).* With regards to the consensus definition, *Rowe et al (1990)* agreed with the proposal that dipstick testing for urine should not be applied to the definition of microalbuminuria *(Mogensen et al, 1985).*

There is a problem is with this consensus definition, with respect to standardization, since most studies have been done on adults. There is need to standardize the definition of microalbuminuria in

relation to sex, age, posture, diurnal variations, methods of sample collection, assay condition and unit of expression for microalbuminuria (*Metcalf and Scragg, 1994; Mortensen, 1994).*

Cembrowski (1990) reviewed 13 publications on the modes of urine collection and units of expression for AER and made the following observations: 4 publications had recommended the use of FMU and albumin concentration; 3 recommended FMU and ACR; 3 recommended the overnight urine collection and ACR; 2 recommended the overnight urine collection and albumin concentration; 2 recommended the timed overnight urine collection and AER; 1 recommendation for random within-day collection and ACR and 1 recommendation for second day urine collection and ACR.

Due to the non-uniformity of collection, there are different definitions of microalbuminuria. These differences may arise from both clinical and analytical procedures for the micro albumin assay (*Rowe et al, 1990*). However, in order to maximize the diagnostic yield of microalbuminuria determination, a timed urine collection can be used for measuring albumin and creatinine concentrations. In addition, information on the adequacy of urine collection (using creatinine excretion rate), type of sample collection preferably 24-hour urine and results expressed as AER, ACR and albumin concentration. The 24-hour urine collection is ideal for the quantitation of protein/albumin excretion, but it has some inherent problems. It is tedious, cumbersome, time consuming, results in delay in diagnosis and 30% of collections are inaccurate. In addition to the foregoing problems, there are some confounding factors that need to be considered before obtaining samples for the diagnosis of microalbuminuria (see Table 1.2).

Table 1.2: Confounding factors that affect Albumin Excretion Rate.

Factor	Albumin Excretion Rate	
	Increased	Decreased
Heart failure	+	
Excessive dietary Protein intake	+	
Exercise	+	
Hematuria	+	
Uncontrolled BP	+	
Uncontrolled diabetes	+	
Perineal contamination	+	
Fevers	+	
Malnutrition		+
ACE inhibitors therapy		+
NSAID		+

ACE-Angiotensin converting enzyme; NSAID-Non-steroidal anti inflammatory drug

1.2.2.2: Diagnosis of microalbuminuria

(i) Microalbuminuria is diagnosed when 2 out of 3 timed urine collections have AER within 20-200μ g/min independent of collection *(Rowe et al, 1990).* It must be noted that AER is 25% lower with

overnight compared with 24-hour collection (*Tomaselli et al, 1989),* and that the upper limit of normal varies with collection procedure.

(ii) 2 out of 3 collections within 6 months form the basis of diagnosis.

1.2.2.3: Prevalence of microalbuminuria.

The prevalence of microalbuminuria varies in terms of sampling procedure, sample size, duration of study, assay method, cut-off levels for albumin excretion and ethnicity. In the USA, the prevalence is higher among Native-Americans, African-Americans, Hispanics and Polynesians than Caucasians of European decent *(Collins et al, 1989; Goldschmind et al, 1993; Gohdes et al, 1993; Nelson et al 1993a; Dasmapatra et al, 1994; Simmons et al 1994, Nelson et al, 1995; Robbins et al, 1996;).* In Africa, studies done in Mauritius, Algeria and Nigeria reported different prevalence for microalbuminuria. The Mauritius study reported a prevalence of 67% in type 2 diabetics *(Hemraj et al, 1995)*. The Algerian study reported a prevalence of 67% and 70% for microalbuminuria for both Type 1 and 2 diabetes respectively *(Maiza et al, 1996; Gill, 1997)*. The Nigerian study was not specific to albumin measurement, rather total protein excretion was measured in the diabetic subjects that included cases of urinary tract infection and reported a prevalence of 42% as having diabetic kidney disease. (*Arijo et al, 1988).*

1.2.2.4: Mechanism(s) of Microalbuminuria.

The major cause of morbidity and mortality in diabetes mellitus is vascular disease affecting both large blood vessels (macroangiopathy) and small blood vessels (microangiopathy). Diabetes mellitus causes specific microangiopathic changes characterized by (i) an increased basement membrane thickening, (ii) increased capillary permeability and (iii) increased microthrombi formation. These

changes are usually generalized with major clinical effects on the kidneys, retina, peripheral nerves, myocardium and the skin *(Jenning and Barnet, 1988).* The pathogenesis involves many factors, including functional abnormalities within the microcirculation, physiological effects of enhanced glucose metabolism via different non-glycolytic pathways, humoral and genetic susceptibility.

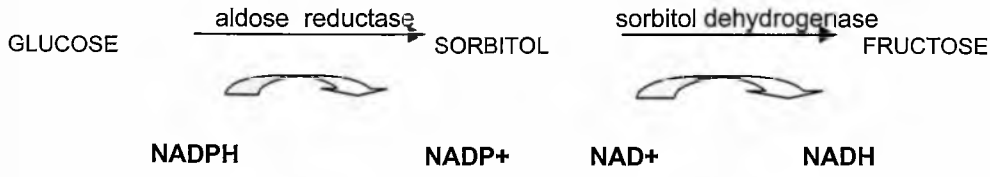
Prolonged hyperglycemia is positively associated with vascular disease, especially in those with prolonged disease duration. Long-term glycaemic control to normoglycaemia may retard or prevent the progression of kidney, retinal and peripheral nerve damage as reported by the Diabetes Complications Control Trials–(DCCT), *(Dahl-Jorgensen et al, 1986).* The actual mechanism of microangiopathy is far from clear.

The common causes of both macro- and microangiopathy are endothelial cell changes, increased oxidative stress due to the presence of "free radical activity" and changes in platelet function. The associations between macro- and microangiopathy with hyperglycemia suggest a common patho-physiological process induced by systemic non-enzymatic glycation of proteins. Glucose is chemically bound to an amino group in proteins to form a Schiff base, which undergoes a slow chemical rearrangement to a more stable AMADORI product. Glucose binding to proteins of higher turnover is reversible, but this is not so with proteins with slower turnover rates physiologically. Amadori products slowly undergo series of irreversible rearrangements and dehydrations to form advanced glycated end (AGE) products resulting in a brownish pigmentation, fluorescence and extensive cross-linkages. This process occurs with slow turnover proteins as found in collagen and basement membranes *(Vlassara, 1996).* One of the AGE products discovered in diabetic tissue is 2-furoyl - 4 (5) - (2 furanyl)-1-H-imidazole. On the other hand, glucose can reduce molecular oxygen in the presence of some trace elements to produce free radicals *(Taniguchi et al, 1996).*

Free radicals are chemical species capable of independent existence. They contain one or more unpaired electrons in its outer orbit. They are derived from molecular oxygen and are very powerful oxidants in body tissues. These oxidants are formed by auto-oxidation of glucose and or during the formation of Amadori products. The rate of production of free radical species depends on the rate of non-enzymatic protein glycation and therefore on the levels of glucose concentration. Free radicals are highly reactive and can be cytotoxic by reacting with DNA to form 8-hydroxy-guanine residues. They can also denature, aggregate, cross-link proteins and oxidize lipids to lipid peroxides. Glycation and oxidation are inextricably linked, for example, low-density lipoproteins (LDL) is highly oxidized by prolonged exposure to glucose. The formation of AGE products also produces free radicals. This process also enhances the formation of free radicals. Oxidative stress occurs when there is an imbalance between production of oxidants and the ability to scavenge the oxidants. There are widespread defensive mechanisms both intra- and extra-cellular. The super oxide dismutase (SOD) is an enzyme whose substrate is super oxide, it is widespread in the body, but its activity is impaired by non-enzymatic glycation of the enzyme. Also, reduced gluthathione ((GSH) detoxifies organic peroxides to produce oxidized gluthathione (GSSG) that is rapidly reduced back to GSH using nicotinamide dinucleotide phosphate (NADPH) as co-factor for aldose reductase. This may become insufficient in chronic hyperglycemia.

In insulin resistant tissues, hyperglycemia enhances the expression of the enzyme aldose reductase which then converts glucose to sorbitol *(Cogan, 1984)* as shown in Fig 1.0.

Fig 1.0: THE POLYOL PATHWAY



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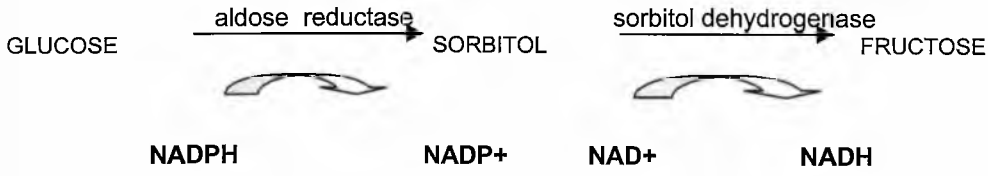


The increased consumption of NADPH through the polyol pathway leaves insufficient NADPH to re-generate anti-oxidants such as GSH or ascorbic acid (Vitamin C). This leaves tissues classically affected by diabetes vulnerable to the action of free radical mediated injuries. Evidence exists in diabetes, where there is an increased production of free radicals and a decline in anti-oxidant capacity preceding diabetic complications.

Increased vascular permeability is a key feature in the diabetic microangiopathic process, and this is also induced by AGE product *(Vlassara, 1996),* resulting in cellular damage to specific cell types. This process is inhibited by GSH and is dependent on the metabolic activity of Cytochrome P-450 enzymes. The vascular permeability depends on cellular integrity and ionic charge of proteoglycan molecules in the glomerular basement membrane. Studies have confirmed that there is leakage of anionic macromolecules e.g. albumin in the urine of diabetic subjects. There is a decrease in the polyanionic proteoglycans content of the glomerular basement membrane (GBM). This arises from reduced binding of heparan sulfate to laminin due to non-enzymatic glycation of the proteins in the GBM (*Kefalides, 1974)*. In the GBM, there are cross-linkages of AGE products that decrease the vascular elasticity and entrap extra-vasated proteins *(Monnier et al, 1996)*. Advanced glycated end products are less susceptible to proteolytic enzyme activity, which leads to further accumulation of AGE products, membrane thickening with progressive luminal occlusion as seen in diabetic glomerulopathy. Similarly, collagen AGE products trap LDL that is often oxidized and may also contribute to the acceleration of atherosclerosis in the diabetic patient. Physiologically, aged collagen and basement membrane are degraded and replaced at a constant slow rate throughout life and this is dependent on macrophage function. Its efficacy will determine the rate and extent of vascular damage. In the diabetic subject the macrophage function is decreased significantly by either metabolic and/or genetic factors.

1.2.2.5 Pathogenesis of Microalbuminuria.

Another patho-physiological mechanism involved in increased AER is the combination of hemodynamic, metabolic changes, genetic susceptibility and humoral factors. The kidney excretion of a substance represents a balance between glomerular filtration, tubular reabsorption and tubular secretion. It is unlikely that there is any active secretion of albumin occurring in the kidney. Filtration and re-absorption determine the excretion process. The GFR, molecular size and charge influence filtration of albumin in the glomerulus. Studies from experimental animals and in early disease in man confirm that 95% of filtered albumin is reabsorbed (*Campbell, 1995).*

The central features of diabetic angiopathic complications are abnormal leakages of albumin (increased trans-capillary escape rate of albumin–TER_{alb}) and luminal constriction of the capillary. Abnormalities of capillary basement membrane including the GBM have been extensively studied in Type 1 diabetes namely; mesangial composition and size *(Wahl et al, 1982;Klein et al, 1986 Shimomira et al, 1987).* Other studies have also indicated that mesangial expansion plays a critical role in the pathogenesis of diabetic kidney disease *(Lee, 1995).* The mesangial matrix early in diabetes is identical to those present in normal glomeruli *(Wahl et al, 1982),* suggesting that in prolonged diabetes there is accumulation of mesangial matrix due to overproduction of matrix component, decreased catabolism or both. This phenomenon is generalized and not kidney specific.

The GBM consists of primarily type IV collagen, hyaluronic acid, heparan sulfate proteoglycan (HSPG) and laminin the major non-collagenous protein. Endothelial cells synthesize heparan sulfate, it is highly anionic in charge *(Comper and Glasgow, 1995; Osicka and Comper, 1995).* Its synthesis in diabetes have been found to be decreased in both humans and experimental animals *(Kanwar and Farquhar, 1979;Wilkinson et al, 1989).* Heparan sulfate is significantly excreted in diabetic

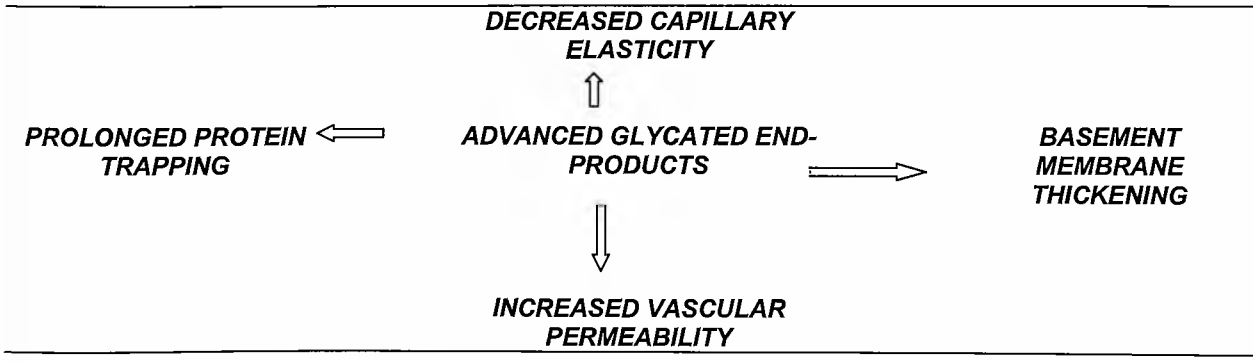
patients *(Groggel et al, 1988;Shield et al, 1995).* The decrease in HSPG is due to less incorporation into the GBM by ligand binding to laminin (*Campbell, 1995*). Glomerular hyper-filtration is present at the onset of diabetes, which then decreases to normal when hyperglycemia is controlled. The glomerular hyper-filtration is due to increased renal blood flow, raised intra-glomerular pressure and increased filtration surface area. Size selectivity studies have confirmed loss of charge selectivity prior to loss of size perm-selectivity in the diabetic kidney *(Viberti and Keen, 1984; Brenner et al, 1978; Scandling and Myers, 1992; Osicka and Comper, 1995; Nakamura and Myers, 1988). Nakamura and Myers (1998)* in a study conducted on Pima Indians with recent onset of Type 2 diabetes noted the loss of glomerular capillary wall size selectivity with an increased frequency of larger pores, which are most likely permeable to albumin, compared to subjects with normal glucose tolerance.

Genetic factors have also been implicated with the pathogenesis of diabetic kidney disease. About 30-40% of Type 1 diabetics is susceptible to the development of overt albuminuria. Following siblings with Type 1 diabetes a familial clustering of diabetic kidney disease have been demonstrated *(Mathiesen, 1993).* However, studies in mono- and di-zygotic twins concordant for diabetes are currently not available. Other factors associated with genetic susceptibility are the presence of essential hypertension, elevated sodium-lithium counter-transport, HLA - DR3 and DR4, polymorphism of the angiotensin-converting enzyme (ACE) and the metabolism of heparan sulfate proteoglycan *(Yoshida et al, 1996).*

Humoral mechanisms have been suggested to play critical roles in the pathogenesis of diabetic nephropathy. There is evidence to the effect that the pleitropic cytokine – transforming growth factor β1 (TGF-β1) levels is elevated in diabetes. More so, the intraglomerular TGF-β1 messenger- RNA was positively associated with the staining intensity of collagen IV in the mesangium, GBM and

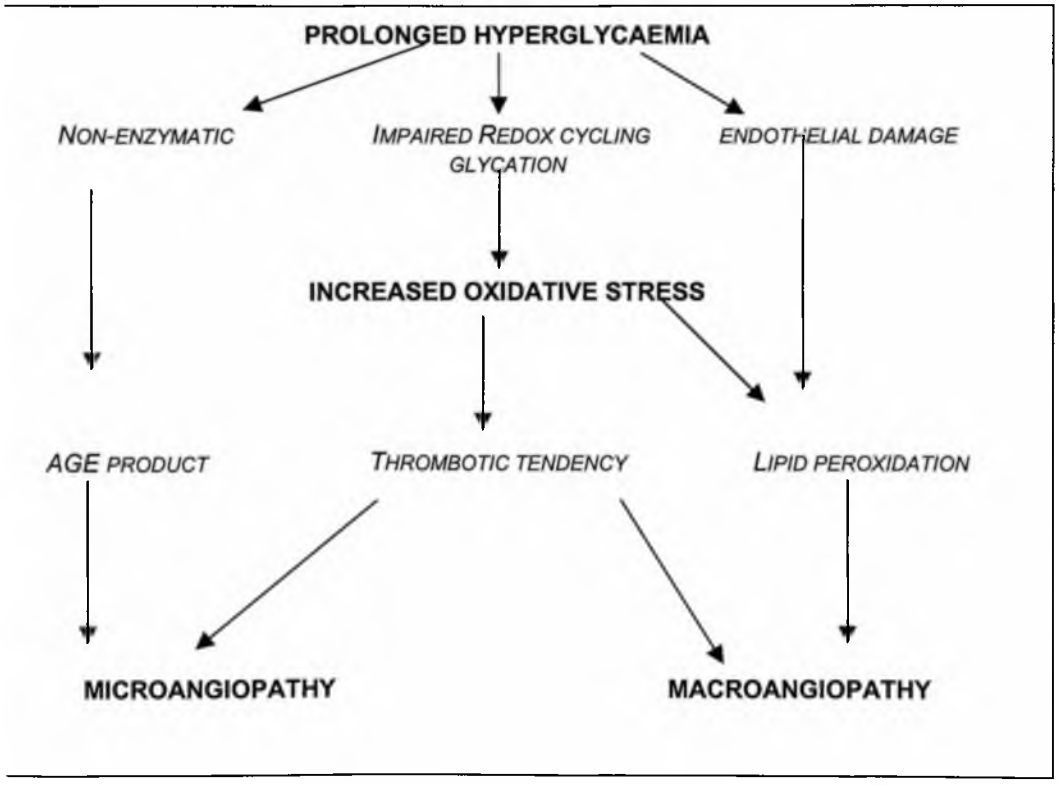
Bowman's capsule *(Sharma and Ziyadeh, 1995; Ziyadeh and Sharma, 1995; Poicet et al, 1998).* Experiments done on laboratory animals showed an increase in TGF-β1 gene expression when cultured mesangial cells were exposed to high glucose concentrations *(Ihm et al, 1995).* The TGF-β 1 regulates almost every known molecule of the extra-cellular matrix (collagen, fibronectin, proteoglycans and tenascin). The TGF-β1 plays a key role in a number of pathological processes that involves the extra-cellular matrix. In diabetes the key features of kidney disease are altered composition of extracellular matrix, GBM thickening and mesangial expansion *(Steffes et al, 1989).* Therefore, hyper-secretion of TGF-β1 triggered by diabetes might be a major contributory factor to the genesis of diabetic nephropathy. Also implicated in the pathogenesis of diabetic nephropathy is endothelial cell dysfunction. There are convincing data from animal and *in-vitro* models, that endothelial cell dysfunction is related to hyperglycemia such as activation of Protein Kinase C, increased expression of TGF-β1, non-enzymatic glycation and oxidative stress. Its relevance to man is unknown as there are no extensive studies in man *(Steuhouwer et al, 1992; Stroes et al, 1995; Steuhouwer et al, 1997).*

Fig 1.1: Possible Role of Advanced Glycated End Products in Diabetic Microangiopathy



Apart from the vascular permeability and basement membrane thickening as seen in diabetic kidneys there is evidence for a reduced endothelial cell production of prostaglandin, nitric oxide which are activators of fibrinolysis together with an increase in von-Williebrand factor. This is exacerbated by an increase in lipo-peroxides, which modulate the Arachidonic acid cascade by inhibiting prostaglandin synthase, thus enhancing micro-thrombus formation *(Ignora, 1996).* Endothelial cell damage due to free radicals activates cyclo-oxygenase enzyme resulting in an increased thromboxane A_2 formation (*Warso and Lands,1983).* Figure 1.2 is a sketch of the mechanism involved in diabetic angiopathy.

Fig 1.2: Sketch of the Mechanisms Involved in Diabetic Angiopathy



1.2.2.6: Natural history of diabetic kidney disease.

Thirty to forty-five percent (30-45%) of Type 1 diabetes will ultimately develop clinical or overt diabetic kidney disease. The onset of clinical diabetic nephropathy is preceded by a decade or more by hyper-filtration, followed by a progressive increase in AER quantifiable by a specific albumin assay systems for example immunochemical methods. The microalbuminuric phase is associated with little or no structural changes and is referred to as incipient diabetic nephropathy. When AER increases into the measurable range by conventional dipstick test >200μg/min, it is referred as "macroalbuminuria" The macroalbuminuric phase is characterized by a progressive decline in GFR and unequivocal histological evidence of diffuse or nodular glomerulosclerosis.

Macroalbuminuria heralds the onset of clinical or overt diabetic nephropathy *(Rowe et al, 1990)*. *Mogensen et al (1983)* described the natural history of diabetic kidney disease into 5 stages in relation to Type 1 diabetes. That of Type 2 has been difficult to characterize. The problem with the latter is that the actual onset of Type 2 diabetes is unknown. It frequently occurs in middle and older aged individuals. Within this age group are some confounding factors like the existence of declining GFR, increased frequency of co-existence of non-diabetic kidney disease beyond age 50 years, and high mortality from cardiovascular disease (*Mogensen et al, 1984)*. This increase in mortality from cardiovascular disease perhaps limits the full manifestation of the natural history of diabetic nephropathy in Type 2 diabetes accounting for 3-8% of all cases progressing to ESRD. *Gall (1997)* compared Type 2 diabetic patients with non-diabetic kidney disease and diabetic glomerulopathy and found a rapid decline of GFR in those with non-diabetic renal disease. Macroalbuminuria is a relatively late manifestation of diabetic nephropathy, promptly followed by the appearance of hypertension and declining kidney function with structural changes (Table 1.3).

Table 1.3: The Natural History of Kidney Disease in Type 1 Diabetes.**

STAGE	ONSET	Functional Anomalies	Structural Abnormalities	Risk factors for progression	% Progress to Next stage
1. Early hypertrophy and hyperfunction Reversible by glycaemic control	At diagnosis	↑GFR; ↑glomerular pressure	↑ Kidney size; ↑ Glomerular volume ↑ Capillary fraction surface area	Hyperglycemia	100
2. Kidney lesions but no clinical signs (Reversible)	2-3 after diagnosis	↑GFR, ↑glomerular capillary pressure albumin not detectable unless marked hyperglycemia and glycosuria are also present	↑ Thickness of glomerular and tubular capillary basement membrane; ↑mesangial volume; early glomerulosclerosis	Hyperglycemia Glomerular capillary pressure; genetic factors ? Hypertension ?? High protein diet	35-40
3. Incipient nephropathy (Reversible)	7-15 after diagnosis	↑AER (30-300mg) /day; normal to slight ↑ GFR, but decline starts	More glomerulosclerosis	Hypertension; ? Hyperglycemia ?? High Protein	80-100
4. Clinical diabetic nephropathy (Irreversible)	10-30yr	AER >300mg/day Normal or slightly increased ↑GFR but declining is progressive	Diffuse glomerulosclerosis	Hypertension ? High Protein diet	75-100
5. End Stage Kidney disease	20-40yr after diagnosis	GFR↓ <10ml/min; serum creatinine ≥ 884μmol/L	Kidney fibrosis		

** Reproduced from Selby et al (1990) and Mogensen et al (1983) GFR – glomerular filtration rate; ↑ -increase; ↓ decrease AER – albumin excretion rate

Several factors have been implicated in the progression of nephropathy through epidemiological, clinical and experimental studies in both human and experimental animals. They are namely; glycaemic control, presence of micro vascular changes, obesity, lipid abnormalities and other non-modifiable factors. It is important to prevent the progression of early diabetic nephropathy to end-stage renal failure i.e. stage 1 to stage 5. There are 3 strategies for its prevention.

Primary Prevention: to prevent progression to stage 2 by screening for microalbuminuria. Interventions required are glucose control surveillance and prompt treatment for hypertension, restriction of dietary protein, identification of persons at risk and screening for retinopathy.

Secondary Prevention: to stop or slow the progression to stage 4. Interventions required are glucose control, blood pressure control and dietary protein restriction.

Tertiary Prevention: to prevent progression to stage 5 i.e. clinical proteinuria to ESRD. Intervention requires rigid blood pressure control and dietary protein restriction *(Selby et al, 1990; Mogensen et al, 1995).*

The general recommendation is that periodic dipstick testing should be part of diabetic medical care. Positive cases will require quantitation for albumin excretion since it is associated with a high risk of developing ESRD and mortality from cardiovascular disease in both types of diabetes.

1.2.2.7: Screening and Sampling for Microalbuminuria.

Physiologically, urinary albumin excretion is increased during strenuous exercise, erect posture and acute water diuresis. This means that urinary collection methods have to be standardized for both screening and monitoring purposes. Urine samples need not be collected after undue exertion or after acute fluid loading *(Rowe et al, 1990)*. Reference values must indicate the type of urine collection as un-timed namely: FMU, single or random void urine and ambulatory morning collection; and for timed urine collections (2-5hr day time collection, overnight or 24hr collections). Most studies into microalbuminuria used timed overnight or 24-hr samples with reference values quoted for a given population *(Metcalf and Scragg, 1994)*. It is a fact that microalbuminuria predicts nephropathy *(Viberti et al, 1982; Mogensen and Christensen, 1984; Mogensen, 1984; Damsgaard and Mogensen, 1986; Almdal et al, 1994)* in diabetics and predicts cardiovascular disease in both diabetic and non-diabetic subjects *(Yudkin et al, 1988).* Improved glycaemic and blood pressure control retards the progression from intermittent to persistent microalbuminuria.

Screening for a disease within a population requires that its outcome can be improved on treatment *(Schwab et al, 1992; Marshall, 1991)* and the test available must be simple, easy to perform, reliable, accurate and inexpensive. Table 1.4 shows various screening studies that used timed urine collection in calculating the AER.

Table 1.4: Studies Showing the Predictive Value of Microalbuminuria.

	Mogensen and Christensen (1984)	Viberti et al (1982)	Mathieson et al (1984)	Parving et al (1982)
No. of patient	43	63	71	23
Follow up (yr)	7	14	6	6
Sample	Short day time	Overnight	24 hour	24 hour
Assay Method	RIA	RIA	RID	RID
AER, Ref range	≤ 7.5 μg/min	< 12μg/min	< 20 μg/min	≤ 40mg/24hr
Cut off AER	15 μg/min	30 μg/min	70 μg/min	40 mg/min
No. >Cut off	12/14	7/8	7/7	7/7

AER - Albumin excretion rate; RIA – Radioimmunoassay; RID – Radial Immunodiffusion

Timed urine collection is ideal for the quantitation of albumin excretion in diagnosing patients with various forms of renal disease. Timed overnight and 24 hour urine collection have been referred to as the "gold" standard for albumin quantification. Timed urine collections suffers from diurnal variations, it is cumbersome and inaccurate in 33% of collections. The use of urinary creatinine concentration to correct for urine flow delays the diagnostic time of diabetic nephropathy *(Rowe et al, 1990)*. To overcome such problems, much emphasis have been placed on the use of single urine collection i.e. FMU, ambulatory daytime sample, random urine or single void urine to improve compliance and standardization of sample collection *(Marshal, 1991)*.

Gatling et al (1985) reported a correlation coefficient of (r = 0.45) between UAC and AER. At a cut-off value >25mg/Lthe sensitivity was 56% and specificity of 81% in predicting AER > 30μg/min. *Kouri et al (1991)* reported a sensitivity of 70% for IDDM and 60% for NIDDM at an albumin concentration cut off value of 20mg/L. On lowering the UAC to 10mg/L, the sensitivities increased to 91% and 87% in IDDM and NIDDM respectively without change in the specificity and a marked improvement in positive predictive value (PPV) with reduced false positives. *Nathan et al (1987)* used an index of 24-hr proteinuria and reported sensitivity ranging between 55-85% depending on the cut-off value.

Watts et al (1986) in their study of Type 1 diabetics reported 96% sensitivity, 90% specificity for UAC > 15 mg/L predicting AER in timed overnight urine of > 15μg/min. However, when UAC was varied to > 25 mg/L the sensitivity rose to 100% with a lower specificity suffered to 85% predicting an overnight AER > 30μg/min. Some workers have also demonstrated good correlation co-efficient between single void urine and 24 hour protein excretion for the assessment of microalbuminuria and proteinuria in both diabetic and non-diabetic subjects with renal diseases *(Ginsberg et al, 1983, Shaw et al, 1983; Schocett and Daneman, 1988).* The use of UAC for screening microalbuminuria is not appropriate for random urine samples, but *Hutchinson et al (1988)* in their study reported that UAC at a cut-off value of 17mg/L in FMU is useful for screening microalbuminuria. The FMU albumin concentration correlates well with timed overnight and 24hr collections than that of random or single void urine albumin (r = 0.86 – 90.0) as shown in Table 1.5.

Table 1.5: Albumin Concentrations in First Morning Urine Predicting AER in Timed Urine Collection

	Colwell et al, (1986)	Gatling et al, (1985)	Marshall and Alberti, (1985)	Hutchison et al, (1988)
Cut off Alb (mg/L)	> 20.0	> 20.0	> 20.0	> 17.0
Type of collection	24-hr AER>20mg/24hr	Overnight-AER*>30μg/min	Overnight-AER>30μg/min	Overnight-AER>30μg/min
Sensitivity	100%	86.6%	91.0%	96.8%
Specificity	57.0%	97.0%	74.0%	90.9%
Predictive value		71.0%		58.8%
Correlation co-eff.		-		0.90

On the contrary, *Weigman et al (1990)* reported a lower sensitivity and specificity;73.0% and 23.0% respectively for FMU albumin concentration > 30.0mg/L to predict 24-hr AER > 20 μg/min. From Table 1.5 it appears that albumin concentration in FMU correlates well with AER than that of random void urine. *Marshall (1991)* suggested that albumin concentration greater than 17-20mg/L in FMU is more sensitive and a specific predictor of an elevated AER than random void urine albumin concentration. The sensitivity and specificity are significantly less than 100% implying large numbers of false positive and false negative results. This will require a more careful and thorough screening tests in detecting elevated AER. *Mogensen (1984)* measured UAC in an unselected diabetic population and reported that 77% of the subjects had urinary albumin concentration less than 30 μ g/mL. There was no correlation between albumin concentration and duration of disease. Mogensen's conclusion was that for the screening of incipient diabetic nephropathy, the urinary albumin concentration methods are the first step in identifying patients at risk. But he conceded that UAC values alone were not sufficient in themselves for the diagnosis of incipient diabetic nephropathy. Due to variations in individual urine flow he suggested that it would be better to improve the effect of

urine flow by using the ACR for screening elevated AER using FMU (*Mogensen, 1984; Cohen et al, 1987a; Marshall, 1991).* On the basis of correcting the effect of urine flow by using ACR, several studies have fairly good correlations to timed urine collections but with different diagnostic sensitivities and specificities to predict AER > 30 μg/minutes (Table 1.6 and 1.7).

TAB 1.6: Results of Studies using UAC and ACR in Predicting - AER ≥ 30 mg/min in 24hr Urine Collection.

Reference	Sample Type	Measurement	Cut-off point	Sensitivity %	Specificity %
Eshoj et al (1987)	Overnight	UAC	> 20.0	90	98
Nathan et al (1987)	Random	ACR	> 3.4	100	100
McHardy et al (1991)	FMU	ACR	> 2.5	97	82

Table 1.7: Results of Some Studies using UAC and ACR in Predicting AER ≥ 30 μg/min in Overnight Collection.

Reference	Sample Type	Measurement	Alb. Cut-off point	Sensitivity %	Specificity %
Gatling et al (1985)**	RANDOM	UAC	> 25.0	56	81
	FMU	UAC	> 20.0	86	97
Gatling et al (1988)	RANDOM	ACR	> 3.0	80	81
	FMU	UAC	> 20.0	82	96
	FMU	ACR	> 3.5	100	95
	FMU	ACR	> 2.0	96	100
	FMU	ACR	> 3.5	88	99
Marshall and Alberti (1986)	FMU	UAC	> 20.0	91	74
	FMU	ACR	> 3.5	98	69
	FMU	ACR	> 4.5	96	80
Hutchison et al (1988)	FMU	UAC	> 17.0	97	91
	FMU	ACR	> 3.0	97	94
Weigmann et al (1990)	RANDOM	ACR	> 3.4	82	81
Jensen et al (1997)	OVERNIGHT	UAC	> 20.0	58	97
	OVERNIGHT	ACR	> 2.5	73	97
Gilbert et al (1997)	FMU	UAC	> 20.0	93	93

Cut-off value (mg/L); ACR (mg/mmol); FMU -First morning urine; UAC – urinary albumin concentration

In observing Tables 1.6 and 1.7 the choice of screening tests lies between UAC measurement and ACR in a first morning urine sample. In diabetics with microalbuminuria there were significant correlation between ACR and AER in overnight, 24 hour and random void sample *(Rowe et al, 1990)*. Marshall advocates the use of albumin concentration in a FMU than random urine. This is cheaper, ensures compliance among diabetic patients attending the outpatient and has a lower intra-

individual variability. However, other studies do indicate comparable variations whether results are expressed as AER or as ACR. The average intra-individual variation of albumin excretion in both diabetic and non diabetics approximate 40% (*Cohen et al, 1987b)*, and data for diabetic children indicate similar trends of variation *(Rowe et al, 1990)*.

Rowe et al (1990) in their review article reported that there was no consensus recommendation for a particular type of urine collection that is most suitable for the screening of microalbuminuria. But a random ambulatory morning (daytime) or first morning urine samples were popular among several investigators for its reproducibility, convenience, patient compliance and the results expressed as ACR or AER. However, the disadvantages of the creatinine assay are the introduction of an additional error to the assay and an extra cost to the screening program. The urine albumin concentration is not recommended since its excretion is dependent on urine flow and may also misclassify some patients. In general, reference intervals for timed overnight collection are 25% lower than daytime collection due to diurnal effects and posture. In quoting reference values, the sample must be defined, type of survey, sex, age, the percentile value defining the cut-off point from a normal healthy population to discriminate those at risk. The approximate reference ranges in adults are:

(i) 24 hour or daytime sample < 20mg/24hr or AER < 14 μg/min or ACR < 2.0mg/mmol

(ii) Overnight sample AER < 10 μg/min or ACR < 1.5 mg/mmol

Longitudinal studies have established the level of microalbuminuria with the highest prognostic value for diabetic nephropathy to be between 2 and 3 times the upper limit of normal.

Jerums et al (1994) in a position statement on microalbuminuria advocated that screening in Type 1 patients should begin 5 years after onset of disease and in Type 2 diabetes at diagnosis since its

onset is not accurately known. It may be asymptomatic for several years before the manifestation of disease. *Alzaid (1996)* reported a high incidence of retinopathy dyslipidaemia, hypertension, obesity and insulin resistance with microalbuminuria.

1.2.2.8: Methods for Determining Urine Albumin

There are several methods for detecting little increases of urinary total proteins and more specifically albumin. The measurements of urinary protein involve turbidimetric and dye binding methods. Several authors have evaluated these methods (*Pesce and Strande, 1973; Bradford, 1976; Iwata and Nishikaze, 1979; McElderry et al, 1982; Dilena et al, 1983; Lott et al, 1983; Phillipou et al, 1989)*. Turbidimetric methods involve the precipitation of proteins using trichloroacetic acid, benzethonium and benzalkonium chloride – a quaternary ammonium compound measured by photometric methods or nephelometry. The latter gives a better precision. Turbidimetric methods in general have poor precision, poor sensitivity, limited linearity and variable responses to different proteins. The dye binding methods using Coomassie brilliant blue (CBB), Ponceau S dye and Pyrogallol red-molybdate measure absorbance shift of a protein–dye complex. These methods have some drawbacks, though they have a satisfactory precision.

The disadvantages of CBB are non-linearity, staining of cuvettes, reagent instability and difficulties in standardization. These problems arise primarily, due to the complex mixture of proteins in the urine, like the matrix that often yield different results with these methods. To improve upon the response to these dye-binding methods, some workers have used sodium dodecyl sulfate (SDS) in reagents or adjusted the reagent contents. This improves the reagent binding to other proteins like globulins at the expense of binding to albumin and other low molecular mass proteins *(Johnson and Lott, 1978; Read and Northcote, 1981; Orsonneau et al, 1989)*. Although these authors regard these methods

as rapid and inexpensive they are not specific for screening microalbuminuria. Most of them detect total urinary proteins well above the critical cut off value for measuring microalbuminuria and are better suited for clinical diabetic nephropathy (overt proteinuria). The determination of urinary total protein is not sufficient for an accurate diagnosis of early diabetic kidney disease. Therefore, specific methods are required to detect proteins whose excretion signify diabetic kidney disease at its early stages such as albumin, immunoglobulins, transferrin, retinol binding protein (RBP), β_1, and β_2 microglobulins and lysosomal enzymatic activities.

The specific measurement of urinary albumin concentration requires sensitive techniques that are valuable clinically and epidemiological as a public health tool. In 1963, *Keen and Chlouverakis* described a radioimmunoassay (RIA) method that detected very low concentrations of albumin in urine not detectable by conventional reagent strips termed "microalbuminuria". The method was sensitive and reproducible down to very low concentrations requiring less than 1000μL of urine sample and up to 100 assays could be estimated in each run. Due to costs involved with RIA, short shelf life of the reagents and health hazards from radioactivity, non-isotopic immunoassay have been developed such as radial immunodiffusion (RID) assay, immuno-electrophoresis (IE); immunoturbidimetry (IT), fluorescent immunoassay (FIA), enzyme linked immunosorbent assay (ELISA) and immuno-nephelometry (IN). These methods are suitable for rapid analysis of large sample numbers, particularly if an automated spectrophotometer system is available. Most of these methods have been compared to RIA and they give accurate results, good reproducibility, and good correlation co-efficient but vary considerably in complexity and they have a minimum turnaround time of at least 2 hours. The antibody–antigen binding in immunochemical assays is affected by sample matrix that varies from urine to the other *(Rowe et al, 1990)*. Therefore it is suggested that methods for measuring urinary albumin be validated over the pathological range of pH, salt content, glucose and calcium concentrations which occasionally may be encountered. They also recommended a target albumin concentration of < 5mg/L as the lower limit of the working range.

Watts et al, (1986) assessed four immunochemical methods for determining low concentrations of albumin in urine and recommended RID but not ELISA that had large variations in test results. One disadvantage of RID is that it cannot be automated (Table 1.8). Table 1.9 compares 6 immunoassays for measuring urine albumin.

Table 1.8: Comparison of 4 Immunochemical Measurements of Urine Albumin

	RIA	RID	IT	ELISA
Albumin concentration	Equal	Equal	Lower	Lower
% Albumin conc. > 30mg/L	-	100	100	74
Analytical range	1-40mg/L	2.5-40mg/L	2.5-60mg/L	6.2-200µg/L
Random error mg/l	-	38	4.3	7.3
Within-run precision %CV	3	3	3	3
Between-run precision %CV	8-9	5-7	3-10	8-10
Analytical recovery %	93-101	94-97	98-102	92-102
Turn around time	24hr	48hr	6hr	6hr
Capital cost	Highest	Lowest	Intermediate	Intermediate
Operational cost	Low	Low	Highest	Intermediate
Skill required	2+	4+	1+	3+

RID - Radial Immunodiffusion; RIA - Radio immunoassay; IT - Immunoturbidimetry; ELISA - Enzyme Linked Immunosorbent Assay

TABLE 1.9: Six Immunoassay Methods Compared for Measuring Urine Albumin

	Fielding et al (1983)	Chesham et al (1986)	Silver et al (1986)	Kramer et al (1987)	Magnotti et al (1989)	Solomon et al (1992)
Type of assay	ELISA	cEIA	FIA	ELISA	ELISA	ELISA
Detection limit	0.78mg/L	<0.9mg/L	1.5mg/L	16.0mg/L	10.0mg/L	2.0mg/L
Assay range	3 - 10µg/L	0.9 - 200mg/L	5 – 200mg/L	0.06–9mg/L	30-300µg/L	1-25mg/L
Within-run %CV	4	2.3-3.7		5.1-9.2	1.2-14.0	2.7-3.9
Between run %CV	8.8-9.2	6.6-8.9	4.5-8.2	9.6-14.9	6.0-22.0	5.6-6.6
% Recovery	95-104	96-104	93-113	92-116	61-109	98-116
Turnaround time	6-hr	1-2hr	1-2hr	7-hr	2-hr	2-hr
Correlation with Reference method		0.96	0.98		0.995	0.989

cEIA-competitive Enzyme Immunoassay; FIA-Fluorescent Immunoassay; ELISA-Enzyme Linked Immunosorbent Assay

Immunoturbidimetric assays are used frequently to assay for low urinary albumin concentrations with comparable results to RIA. *Teppo (1982)* described a rapid, sensitive immunoturbidimetric assay that correlated well with RID (r=0.99); with a linearity of 5-400µg/L which encompasses both normal and pathological albuminuria. It had an imprecision of 5.6-7.6% for both within and between run co-efficient of variation and the average recovery studies was 110%. *Rowe et al (1990)* on comparing an IT assay to RIA (both in-house and commercial kits at albumin concentrations <20mg/L) showed that the imprecision exceeded 12% and recommended that careful attention be paid to values within this range which represents the cut-off value for microalbuminuria. *Linton and Rowe (1988)* evaluated a commercial IT assay versus an in-house method and reported an imprecision of 1.02-4.84%, 1.89-3.43% and 0.99-3.5% for urine albumin concentrations at low, medium and high concentrations respectively. There was a good correlation with the in-house method but its absorbance's were higher. Thirty percent (30%) of the assays were not measurable with the equipment used.

In addition to the various isotopic and non-isotopic immunoassay methods, a number of screening procedures have been developed; *(Albuscreen,by Cambridge Life Sciences; Microbuminitest by Miles Laboratories and quite recently a dry immunochemical method – Micral Test and Micral Test – II by Boerhinger Mannheim).* The Micral Test is semi-quantitative, is easy to perform and requires little skill with a concordance between 88.3-93.0% from 2 studies. Micral Test is an albumin specific dipstick based on a combined immunological and chromatographic principle. An anti-albumin globulin is labeled with the enzyme α-galactosidase, which reacts with chlorphenol red-galactose causing a change in colors from yellow to red. In the case of Micral Test reading of the test results is time dependent (5 – 6 minutes). The color development becomes deeper after such a time resulting in misclassification of patients with microalbuminuria. The Micral Test II has a gold-labeled antibody that generates a red color on the detection pad. The reaction is complete in 60 seconds and stable for 24hours. Several workers have reported sensitivity of 75 – 100% and specificity of 71 – 97% with a negative predictive value of 82.6 – 98.2%. The Micral Test is therefore a rapid, sensitive, semi-quantitative method, useful as a preliminary test for screening and monitoring increased levels of urinary albumin concentrations. It is however not useful for stored urinary samples, and moreover it is time dependent. Micral Test II is however a better test than Micral Test (*Bangstad et al, 1991; Marshall et al, 1992; Jury et al, 1992; Tiu et al, 1993; Mogensen et al, 1997). Tiu et al (1993) and Mogensen et al (1997)* reported a prevalence of microalbuminuria as 28% and 52% respectively using Micral Test II. *Gilbert et al (1997)* assessed Micral Test II against RIA and reported an overall sensitivity and specificity as 93% with a positive predictive value of 89%. They recommended its usefulness for screening microalbuminuria. However they did not however recommend it for diagnosis of microalbuminuria since a positive test required confirmation with timed urine collection for measuring AER using a laboratory base method. *Tui et al (1993)* reported 100% sensitivity using Micral Test II. But the inter-observer variation at albumin concentration at 32mg/L was very high, confirming the misclassification of microalbuminuria using a cut-off value of 20mg/L.

Fluorescent methods are better than absorptiometry or reflectometry in terms of detection limits even when dealing with turbid samples. Using laser excitation sources enhances the detection limit of fluorescent assay further. *Kessler et al (1992)* described a non-immunological method that was simple, rapid, robust and accurate based on a fluorescent probe *(Albumin Blue 670)*, which fluoresces on binding to albumin. Its working range was between 1-100mg/L, which covers both normal and pathological urine albumin concentrations. It has a day-to-day imprecision of < 7% and within–run imprecision of < 4% and correlates well with a Behring nephelometric assay (r= 0.99) for n=100. Proteins such as IgG, Bence-Jones proteins and Transferrin do not interfere with the test. The Albumin Blue 670 (AB 670) has a poor stability and short shelf - life of about 6 weeks. A new derivative of AB 670, developed as Albumin Blue 580 (AB 580), has a better stability for at least 24 months at +4°C when stored in the dark and have been used to assay serum albumin *(Kessler et al, 1997a; Zerbert et al, 1996).* In the study done by *Kessler and associates (1997a)* they reported a correlation co-efficient with Behring Nephelometry as r=0.922 ± 0.010; with an optimum performance for the assay at pH 7.4. The AB 580 has a relatively weak fluorescence in the absence of albumin but the fluorescence increases 17-fold upon binding to albumin. The Albumin Blue probes are anionic in nature and binds to albumin in a specific way to undergo strong fluorescence. There is no significant interference from other proteins. They are exceptionally sensitive for albumin and its binding is instant. The assay is adaptable for general application using a conventional spectrofluorometer *(Kessler et al, 1997b).*

1.2.2.9: Implications of Microalbuminuria

The definition of microalbuminuria varies among different investigators. Although there is a "consensus definition", this is not agreed upon universally. This is partly due to the differences in AER values as reported from various prospective studies with varying thresholds in both Type 1 and

2 diabetics *(Townsend1990)*. Table 1.10 shows studies where microalbuminuria is a strong marker in predicting overt diabetic nephropathy.

Table 1.10: Values of Urinary Albumin Excretion Rate that Predicts later Clinical Nephropathy and/or Mortality in Diabetic Patients.

	Group	Type of diabetes	Predictive urinary albumin excretion µg/min	Type of Urine Collection	Years of Follow up
1.	**Viberti et al (1982)**	1	30	Overnight	14yrs
2.	**Jarret et al (1984)**	2	10	Overnight	14yrs
3.	**Mogensen and Christensen (1984)**	1	15	1-2hr	6-14yr
4.	**Mogensen (1984)**	2	30	Morning	10yr
5.	**Mathieson et al (1984)**	1	70	24hr	6yrs

Exercise induced AER easily manifests itself in diabetics with microalbuminuria as compared to non-diabetics subjects. This rise is so marked that some workers are advocating exercise induced AER to be used for screening microalbuminuria *(Mogensen, 1971; Jefferson et al, 1985)*. Due to effect of physical activity on the 24hr urine collection it is not surprising that this collection is 25% higher than timed overnight urine collection. Microalbuminuria is usually absent at the time of diagnosis of Type 1 but it is highly probable at diagnosis of Type 2 diabetes. It is associated with early onset of diabetes, longer duration of diabetes, poor metabolic control, hypertension, retinopathy, positive family history

of hypertension, high dietary protein intake, sodium-lithium counter-transport, lipoprotein disorders and coagulopathy *(Rowe et al, 1990; Walker, 1991).* Most of these associations have been demonstrated in adult diabetic patients. Little has been reported for the pediatric age group. Only few studies have investigated albumin excretion in the urine of normal children. The studies reported a 95% confidence interval as 7-12.2μ g/min/1.73m² surface areas and a prevalence of microalbuminuria in children with diabetes as 7-20% depending on the cut-off points for urine albumin excretion. Finally, microalbuminuria reflects a generalized increased TER_{alb}*(Feldt–Rasmussen, 1986; Gosling, 1995a*) and which is also a marker of generalized microvascular angiopathy as a result of a generalized endothelial cell dysfunction. In Type 2 and non-diabetic subjects, microalbuminuria predicts an early and excess mortality from cardiovascular causes *(Steuhouwer et al, 1992;Wincour, 1992; Wang et al, 996; Yudkin et al, 1988).*

1.3: OBJECTIVES

1.3.1: Main objective:

To evaluate the non-immunological, non-isotopic Albumin Blue 580 assay for detection of microalbuminuria in Ghanaian Subjects.

1.3.2: Specific Objectives

a. Assessment of diagnostic efficacy namely: sensitivity, specificity and predictive values using AB 580 dye in screening for microalbuminuria.

b. The use of AB 580 to screen for microalbuminuria in the community and clinic diabetic subjects.

c. Identifying associated risk factors for the development of early diabetic kidney disease.

1.4: RATIONAL FOR STUDY

Microalbuminuria represents an early marker of vascular abnormalities including those of the glomerular capillary wall (GCW) *(Yudkin et al, 1988; Damsgaard and Mogensen, 1986; Steuhouwer et al, 1992; Gosling, 1995a & 1995b; Rodico et al, 1998;)* in both diabetic and non-diabetic patients. Microalbuminuria is a prelude to overt nephropathy in diabetic subjects. Currently in Ghana there is no assay for screening urine for microalbuminuria among diabetic subjects. The AB 580 assay is relatively inexpensive, sensitive, specific, accurate, easy to perform, robust and has a good turnaround time. The AB 580 assay has not been validated for screening microalbuminuria in Ghanaian diabetic subjects. In this study, the AB 580 assay would be validated and used to screen for microalbuminuria in diabetic subjects.

The benefits to be derived from this study are:

(a) Primary prevention of microalbuminuria at diagnosis of diabetes, in order to maintain non-progression to stage 2,

(b) Secondary prevention is to reverse or halt progression from microalbuminuria towards clinical nephropathy and

(c) Tertiary prevention level is to delay the onset of end-stage kidney disease. The other benefits to be derived from screening and its application to routine use is to establish a relevant database for research, planning and costing of diabetes care in Ghana.

CHAPTER TWO

2.0 MATERIALS, METHODS AND PATIENT SELECTION

2.1: MATERIALS

Isopropanol, 3-N-Morpholinosufonic free and salt (MOPS), Ethylenediamine tetraacetic acid (EDTA), and Human gamma immunoglobulin (h-IgG) fat free were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Potassium di-hydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) were obtained from British Drug House (BDH) Poole, UK. Human serum albumin solution was from Randox Laboratory Ltd., UK.

AB 580-dye salt was a kind gift from Dr Kessler, Belgium. All other chemicals were obtained in the purest form available.

2.2: METHODS AND PATIENT SELECTION.

2.2.1 ALBUMIN BLUE 580 ASSAY EVALUATION.

The AB 580 assay was evaluated in accordance with the recommended criterion of *Cembrowski and Sullivan (1992)*. 2.5mL of working reagent (Appendix 1.C) were added to 0.5 mL of distilled water as blank, control, undiluted urine and standard albumin solution (Appendix 1.E). The reaction was immediate, and the relative fluorescence emission was measured at excitation wavelength of 610nm and emission wavelength of 630nm in a spectrofluorometer. (SFM – 25, KONTRON INSTRUMENTS, SWITZERLAND)

2.2.1.1 Linearity:

The linearity of the AB 580 dye assay was assessed in duplicates for each level in 2 batches (n = 19 runs) for the 8 standard solutions (Appendix 1-E).

2.2.1.2 Precision:

The precision study was done on 3 urine control samples with low, intermediate and higher albumin concentrations for both within and between- batch co-efficient of variation (CV%) in triplicates for 9 runs.

2.2.1.3 Reproducibility and Recovery Study:

Reproducibility was done on freshly pooled urine with a known albumin concentration of 6.0mg/L to which known concentrations of serum albumin were added to give albumin concentrations of 14.5, 50.2 and 100.0mg/L.The co-efficient of variation was calculated for duplicate assays for n = 3 runs.

Serum albumin was added to urine albumin with low, intermediate and high urine albumin content to give concentrations of 5.2, 12.9 and 86.3mg/L for recovery studies.

2.2.1.4 Effect of storage:

Twenty-six (26) albustix negative urine samples were obtained from newly diagnosed diabetic subjects during NCDS '99 and were examined for the effect of temperature and duration on storage. The samples were stored +4°C, -20°C and -80°C for 14 days, 30 days and 6 months with the assays done in triplicates.

2.2.2: NON-COMMUNICABLE DISEASE SURVEY 1999 (NCDS '99)

Three hundred (300) civil servants aged between 20 to 65 years, mentally sound and non-pregnant (in case of females) from the Ministries of Finance and Health were invited to participate in this survey during the 1999 Civil Service week from June 21 to 25. Two hundred and forty five (245) responded to the invitation. Informed consent, formal and verbal was obtained from subjects. They were briefed by the Field Coordinator to participate in the program, by explaining the purpose of the survey, its objectives and procedures. Willing respondents were asked to fast overnight for 10 -14 hours and report to the survey centre between 07.00 - 10.00 hours the next morning.

On arrival subjects were asked to pass urine and discard. A fasting blood sample was taken, then subjects were asked to drink 300mL of a 75g anhydrous glucose within 5 minutes. During the 2-hr OGTT the subjects were made to rest and respond to a questionnaire administered by trained medical personnel. The height (metres), weight (kilograms), waist and hip circumference (centimetres), and electrocardiogram (ECG) were done. Trained persons recorded the blood pressure. At the end of the 2-hour OGTT another blood sample for glucose was taken. Subjects were asked to pass all urine into 500mL containers. The urine was tested with dipsticks for proteins, blood, nitrite and leukocyte *(Nubenco Diagnostics New Jersey, USA)* and the total volume recorded. Urine samples positive for protein, blood nitrite and leucocytes were excluded from the screening for microalbuminuria. 5.0mL aliquots were put into capped polypropylene tubes and stored on ice at the survey center, then brought to the Diabetes Research Laboratory of the University of Ghana Medical School to be stored at +4°C and the AB 580 assay was performed within 48 hours. Diabetes mellitus and hypertension were diagnosed in accordance to *American Diabetes Association (ADA) Expert Committee–(1998) and The Joint National Committee (JNC-V) of Detection and Treatment of High*

Blood Pressure (1993) respectively. Subjects previously known to have diabetes or hypertension and who were on treatment were classified as such for these conditions.

2.2.3: OUTPATIENT DIABETIC SUBJECTS

Diabetic subjects attending the Management Centre were asked to provide 2 consecutive spot urine samples within 14 days and dipstick tests done on them. Two hundred and twelve (212) patients with albustix negative urine were recruited to provide a single 12-hr overnight urine collection. After verbal and written instructions were given to the patients, 4.0 litre containers containing 2.0mL of sodium azide (5g/L) as a preservative were provided for the overnight urine collection. All the urine collected was brought to the clinic the next morning. The total volume was recorded and aliquots stored at 20°C and assayed within 4 days.

2.3: DATA ANALYSIS

All statistical analysis was carried out with Excel 7 (Microsoft Office 1997). Albumin excretion rates distributions were positively skewed in both NCDs and the outpatient diabetic population. The albumin excretion rate results were logarithmically (log_{10}) transformed before analysis. An unpaired 2 tail Student - t test as appropriate with the 95% confidence levels determined assessed the differences between means.

In calculating the diagnostic efficacy of the AB 580 dye for screening microalbuminuria in the community population, a positive predictive value (PPV) is the proportion of subjects with a positive test that were correctly diagnosed as having the disease. The negative predictive value (NPV) is also

the proportion of subjects with negative results who were correctly diagnosed as not having the disease under investigation. The predictive value of a test in clinical practice depends on the prevalence of the abnormality. If the prevalence is low, the PPV will not be close to 100% even if the sensitivity and specificity are high, therefore in screening a population it is likely that many results will be false positive.

Subjects were diagnosed as diabetics if they had a 2-hour post glucose load plasma glucose concentration ≥ 11.1mmol/L *(ADA-Expert Committee, 1998)*. Hypertension was present if the systolic blood pressure was ≥ 140mmHg and/or diastolic blood pressure ≥ 90mmHg *(JNC-V, 1993)*.

2.3.1 Calculation of Albumin Excretion Rate

Albumin excretion rate (AER) was calculated in microgram per minute using this equation:

$(U_{alb} \times TV) \div T$ = AER µg/min (Timed Collection).

Where U_{alb} is albumin concentration (µg/mL), **TV** -total volume of urine (mL) and **T** - time of urine collection (minutes).

2.3.2: Equations for Calculating the Diagnostic Efficacy

Contingency Table: ***Gold Standard Test for Screening Disease***

Alb. Conc. mg/L	*Disease present*	*Disease absent*	**Total No.**
Screening test positive	**TP**	**FP**	**TP + FP**
Screening test negative	**FN**	**TN**	**FN + TN**
Total No.	**TP + FN**	**FP + TN**	**TP + FN + FP + TN**

Sensitivity (Se) = True Positives (TP) ÷ (True Positive + False Negatives (FN)

Specificity (Sp) = True Negatives (TN) ÷ (True negatives + False Positives (FP)

Prevalence (Pr) = (True Positives + False Negatives) ÷ Population size

Positive Predictive Value (PPV) =(Se. × Pr) ÷ (Se. × Pr) + (1 - Pr) (1 - Sp)

Negative Predictive Value = Sp × (1 - Pr) ÷ (1-Se) (Pr) + Sp (1 - Pr) (**Engelgou** *et al, 1995)*

Pre-test Odds = Pr ÷ (1 - Pr) is the prior probability of a positive test.

Post-test Odds = Pre-test Odds × Likelihood Ratio is the posterior probability of a correct diagnosis.

Posterior probability = Post test odds ÷ (Post test odds + 1)

Likelihood Ratio = Se ÷ (1 - Sp)

Diagnostic accuracy = (TP + TN) ÷ Population size (*Altman and Bland, 1990)*

CHAPTER THREE

3.0: RESULTS:

3.1 EVALUATION OF AB 580 DYE

3.1.1: Linearity.

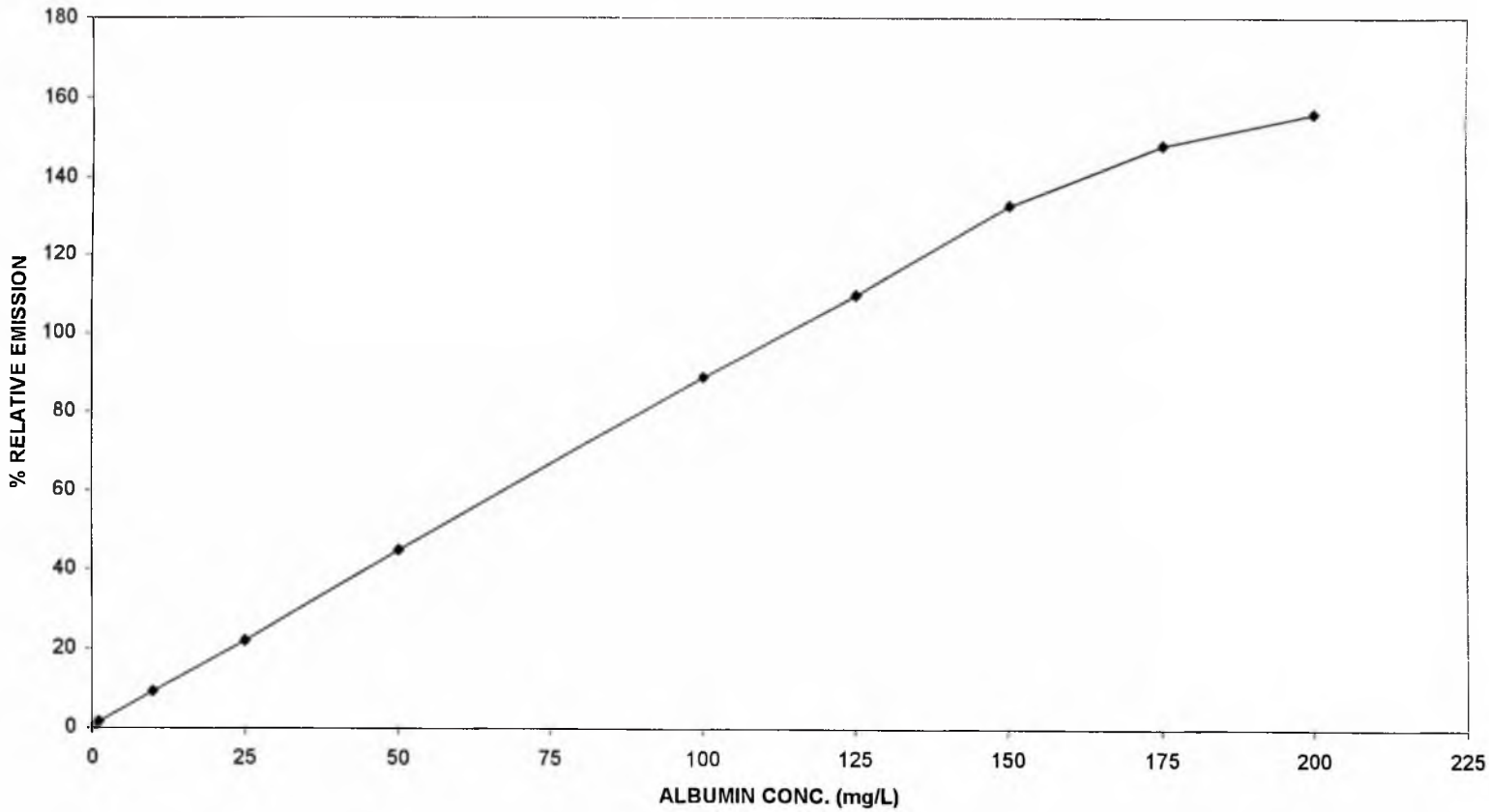
Figure 3.1 is the calibration curve using human serum albumin standard (Appendix 1.E, Tab 1.A). The curve was linear up to 150mg/L. This spans the range of microalbuminuria. It has a minimum detection limit (3SD of blank) for 2 – 200mg/L and 1 – 50mg/L standards as 1.4mg/L and 0.9mg/L respectively. The curve was plotted as the difference between the percentage relative emission and the percentage blank emission as shown in Table 3.1.

Table 3.1: Percentage Relative Emission of Human Serum Albumin Standards.

Alb. Std conc. (mg/L)	% Relative Fluorescence Emission (RFE)	%F=RFE– Blank
Blank	10.0 ± 0.35	0.0
1.0	11.5 ± 0.25	1.5
10.0	19.5 ± 0.75	9.2
25.0	32.0 ± 0.41	22.0
50.0	55.0 ± 0.72	45.0
100.0	99.1 ± 0.22	89.1
125.0	120.0 ± 1.2	110.0
150.0	143.0 ± 0.99	133.0
175.0	158.1 ± 2.2	143.0
200.0	166.0 ± 4.4	156.0

Results are means ± SD for n = 19

FIG. 3.1: CALIBRATION CURVE FOR AB-580 USING HUMAN SERUM ALBUMIN AS STANDARDS



3.1.2: Precision Studies.

Table 3.2 shows the data for intra– and interassay imprecision for 3 urine samples with albumin concentrations of 14.5, 50.2 and 100mg/L (n=19) in triplicates. The intra-assay and inter-assay coefficient of variation – CV% were less than 4% in all cases of albumin concentrations studied.

Table 3.2: Intra-assay and inter-assay Imprecision

Alb. (mg/L)	Intraassay CV%	Interassay CV%
*14.5	3.3	2.7
50.2	3.7	2.6
100.0	1.1	0.6

** Duplicate assay*

3.1.3 Recovery and Reproducibility Studies:

The percentage recovery of added albumin with concentrations of 27.2, 56.0, and 100.0mg/L to 3 urine samples of known albumin content (5.2, 12.9, 86.3mg/L) is shown in Table 3.3. The recovery of the added albumin was 95.6 to 112% for triplicate determinations (n=3).

Table 3.3: Recovery of Added Albumin to Urine Samples.

Initial Alb. (mg/L)	Added alb (mg/L)	Actual Alb. rec.(mg/L)	% Recovery
5.2	27.2	31.8	98.3
	56.0	60.0	98.1
	100.0	109.4	104.2
12.9	27.2	43.7	109.0
	56.0	77.2	112.0
	100.0	112.6	99.7
86.3	27.2	113.4	99.9
	56.0	136.0	95.6
	100.0	183.7	98.7

Results are means for n=3

Table 3.4 shows results of the reproducibility test. Two known amounts of human serum albumin were added to freshly pooled urine samples of a known albumin concentration of 6.0mg/L to give a final concentration of 62.4 and 130.2mg/L. The CV was less than 9.0% for 9 determinations (n=9) for each albumin concentration.

Table 3.4: Reproducibility Study.

Known Alb. (mg/L)	Actual Mean (SD)	CV%
6.0	6.1 ± 0.5	8.2
62.4	63.1 ± 2.9	4.6
130.2	132.8± 6.5	4.9

Results are means ± SD for n=9

3.1.4: Storage of Urine:

Twenty- six (26) albustix negative urine samples from newly diagnosed diabetics were examined for the effect of temperature and duration on storage. Results indicate that albumin concentrations determined at all the 3 temperatures (+4°C, -20°C, and -80°C) within 14 days gave reproducible results. However on freezing for 30 days and 6-mths (at -20°C and -80°C) there was a reduction of 8.5 and 16% respectively in albumin concentration compared to the 14 days storage, although albumin concentrations for the same duration of storage were reproducible (Table 3.5). The differences were not significant; $p > 0.05$.

Table 3.5: Effect of Storage Temperature and Duration on 26 Urine Samples.

Duration; temperature	Albumin (mg/L) mean ± (SEM)	Range (median)	95%CI
< 14 days, / + 4°C	18.9 ± 3.5	1.8 – 84.4 (13.8)	12.0-25.8
< 14 days, / - 20°C	18.6 ± 3.4	1.7 – 83.4 (13.8)	11.9-25.3
< 14 days,/ - 80°C	18.9 ± 3.4	1.8 – 84.1 (13.9)	12.2-25.6
30 day,/ - 20°C	17.4 ± 3.1	1.2 – 80.0 (12.8)	11.3-23.4
30 day, /- 80°C	17.0 ± 3.0	1.2 – 78.0 (13.9)	11.1-22.9
6 month, - 20°C	16.1 ± 3.1	1.0 – 79.0 (12.0)	9.8-22.4
6 month, - 80°C	15.6 ± 3.2	1.0 – 80.0 (12.9)	8.9-22.3

Results are mean ± SEM (n = 3); CI –Confidence interval.

3.2: NON-COMMUNICABLE DISEASE SURVEY:

Two hundred and eighteen (218) subjects provided urine at the end of the OGTT. Twenty-six (26) aliquots had trace or positive proteinuria, 3 were positive for nitrite; 58 subjects were non-diabetic but hypertensive, 32 aliquots were inadequately labeled and were therefore excluded from the screening study. In all 119 subjects were excluded from the study.

Fifty-two (52) subjects who were non-diabetic and non-hypertensive served as the control group with mean age 38.7 (22-55 years). Forty-seven (47) subjects were diabetic with a mean age of 47.3 (23-65years). The age distribution of newly diagnosed diabetic subjects who participated in the survey is shown in Figure 3.2. The mean AER for all the age ranges were above 20.0μg/min. Table 3.6 reveals the characteristics of both control and diabetic subjects. The mean age, systolic blood pressure and AER were significantly lower in the non-diabetic than diabetic subjects ($p < 0.05$).

Figure 3.2 is the graphical presentation of the mean blood pressure for both non-diabetic and diabetic subjects. Both systolic and diastolic blood pressures were higher in the diabetic subjects than the non-diabetic subjects {systolic BP 119.1(10.2)} vrs {137.3(20.0)] mmHg}; {diastolic BP [77.7 (7.5)} vrs {88.6 (13.0)} mmHg.

Table 3.6: Characteristics of both Control and Newly Diagnosed Diabetic Subjects. Mean (SEM).

Characteristic	Control(n = 52)	Diabetics(n = 47)
Male: Female	30:22	26:21
Age (yr)	38.7 ± 8.6 ✳	47.3 ± 9.0
Range (median)	22-55 ± 39	23–65 ± 49
sBP (mmHg)	119.1 ± 10.1✳	137.3 ± 22.0
dBP (mmHg)	77.7 ± 7.5	88.6 ± 13.0
*AER (µg/min)	11.6 ± 1.9 ✳	034.6 ± 2.1
% MAU	14	85
% Hypertensive		55.3

**AER: albumin excretion rate (geometric mean); ✳ p<0.05; sBP-systolic and dBP–diastolic blood pressure; MAU-Microalbuminuria. Results are mean ± SD*

The blood pressure characteristics are presented in Table 3.7 for the diabetic subjects who were normotensive (NT) and hypertensive (HPT). 55.3%(26) of the diabetic subjects were hypertensivse and had systolic blood pressure of ≥ 140 and /or ≥ diastolic blood pressure 90 mmHg. The AER was significantly lower in the normotensive than the hypertensive diabetic subjects p< 0.05; 35.2 ± 5.37 vrs 56.5 ± (10.6) µg/min. A similar significant difference existed between non-diabetic subjects and normotensive diabetic subjects (p < 0.05), (11.6 ± 1.9 vrs 35.2 ± 5.37) ug/min. The mean systolic pressure in the normotensive diabetics subjects was significantly higher than the non-diabetic subjects; p < 0.05 (Table 3.8).

Table 3.7: Blood Pressure Characteristics of the Diabetic Group from NCDS Survey,

Characteristic	NT (n = 21)	95%CI	HPT (n = 26)	95%CI
Male: Female	10:11		16:10	
Age (y)	46.5 ± 22.5 *	43.2-52.0	47.0 ± 1.7	43.7-50.3
Range (median)	23 –59 (50.5)		33 – 65 (47.0)	
sBP (mmHg)	122.2 ± 2.22*	117.8 –126.6	148.6 ± 4.38	148.6-157.2
Range (median)	106 –138(125)		118.0-220.0 (148)	
dBP (mmHg)	79.2 ± 1.67	75.9 – 82.5	86.6 ± 1.9	82.8-90.4
*AER µg/min	35.2 ± 5.37*	24.7 – 45.7	56.5 ± 10.6	35.7-77.3
Range (median)	11.9 – 88.6(25.1)		5.4 –265.2(48.2)	

** p < 0.05 ; NT-normotensives; HPT-hypertensives.* AER (geometric mean); CI – Confidence Interval, Results are means ± SD .*

Table 3.8 Albumin Excretion Characteristics of Non-diabetic Subjects and Normotensive Diabetic Subjects.

Characteristics	Non-diabetic subjects	Normotensive diabetic subjects
Number	52	21
Male: Female	30:22	10:11
Age Range (median) years	22 – 55 (39)	23 – 59 (50.5)
***AER (µg/min)**	11.6 ± 1.9	35.2 ± 5.37*
sBP (mmHg)	119.1 ± 10.1	122.2 ± 22.2

**AER Albumin Excretion Rate - Log_{10} transformed; Mean ± SD; * p< 0.05*

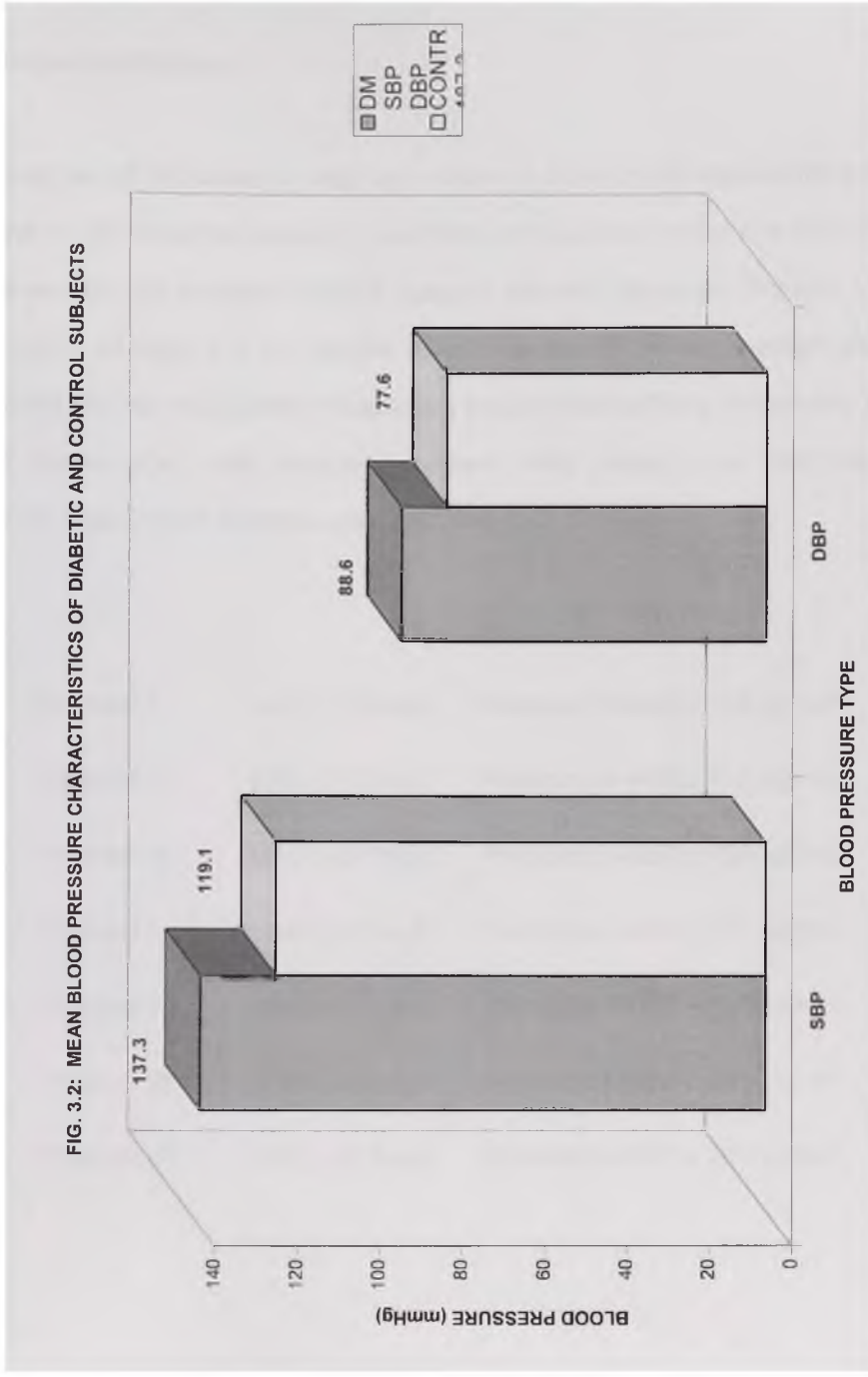


FIG. 3.2: MEAN BLOOD PRESSURE CHARACTERISTICS OF DIABETIC AND CONTROL SUBJECTS

3.2.1: Diagnostic Efficacy.

In assessing the AB 580 assay for diagnostic efficacy in screening for microalbuminuria, 7 proposals were made to determine the sensitivity, specificity and predictive values in a community population (NCD-survey '99) and outpatient diabetic subjects attending the clinic. Proposal VI with a cut-off value of UAC > 25.0mg/L in a 2-hr daytime collection predicting 12-hour overnight albumin excretion rate (N-AER) was the most suitable in assessing the diagnostic efficacy *(Mogensen, 1983; Gatling et al, 1985; Colwell et al, 1986; Marshal and Alberti, 1986; Gatling et al, 1988; Kouri et al, 1991; Marshal and Alberti, 1991; McHardy et al, 1991; Marshall, 1996).*

Proposal 1:	UAC ≥ 10.0mg/L	Predicting N-AER ≥ 15.0 μg/min
Proposal II:	UAC ≥ 15.0mg/L	Predicting N-AER ≥ 15.0 μg/min
Proposal III:	UAC ≥ 20.0mg/L	Predicting N-AER ≥ 15.0 μg/min
Proposal IV:	UAC ≥ 25.0mg/L	Predicting N-AER ≥ 15.0 μg/min
Proposal V:	UAC ≥ 30.0mg/L	Predicting N-AER ≥ 15.0 μg/min
Proposal VI:	UAC ≥ 25.0mg/L	Predicting N-AER ≥ 20.0 μg/min
Proposal VII:	UAC ≥ 30.3mg/L	Predicting N-AER ≥ 20.0 μg/min

Table 3.9 Contingency Tables for Proposals I – VII:

		CONTROLS			DIABETICS		
Proposal I	**UAC mg/L**	**N-AER µg/min**		**Total**	**N-AER µg/min**		**Total**
		≥15.0	**<15.0**		**≥15.0**	**<15.0**	
	≥10.0	21	18	**39**	41	5	**46**
	<10.0	1	12	**13**	1	0	**1**
Total		**22**	**30**	**52**	**42**	**5**	**47**
II		**≥15.0**	**<15.0**		**≥15.0**	**<15.0**	
	≥15.0	21	8	**29**	41	3	44
	<15.0	2	21	**23**	1	2	**3**
Total		**23**	**29**	**52**	**42**	**5**	**47**
III		**≥15.0**	**<15.0**		**≥15.0**	**<15.0**	
	≥20.0	16	6	**22**	40	2	**42**
	<20.0	6	24	**30**	1	4	5
Total		**22**	**30**	**52**	**41**	**6**	**47**

Proposal		N-AER µg/min			N-AER µg/min		
IV	UAC(mg/L)	≥15.0	<15.0	Total	≥15.0	<15.0	Total
	≥25.0	10	5	15	41	0	41
	<25.0	12	25	37	1	5	6
Total		22	30	52	42	5	47

V		≥15.0	<15.0		≥15.0	<15.0	
	≥30.0	10	3	13	36	0	36
	<30.0	12	27	39	6	5	11
Total		22	30	52	42	5	47

VI		≥20.0	<20.0		≥20.0	<20.0	
	≥25.0	4	6	10	40	1	41
	<25.0	2	40	42	0	6	6
Total		6	46	52	40	7	47

VII		≥20.0	<20.0	Total	≥20.0	<20.0	Total
	≥30.0	4	8	12	36	3	39
	<30.0	5	35	40	1	7	8
Total		9	43	52	37	10	47

Table 3.10 shows a summary of the diagnostic efficacy for the 7 proposals in predicting microalbuminuria in the diabetic subjects and absence of microalbuminuria in non-diabetic subjects. In Proposal VI, UAC of ≥ 25mg/L as a cut-off value in predicting N-AER > 20.0 μg/min, the diagnostic sensitivity and PPV were 100% and 97.5% respectively among the diabetics and a specificity of 87% for the absence of microalbuminuria among the non-diabetic subjects. The negative predictive value was 100%. On increasing the cut-off value of UAC to 30mg/L, the sensitivity and PPV decreased among the diabetic with more false positive tests. The specificity and NPV also decreased in the non-diabetic group with more false negative tests. The prevalence of microalbuminuria for proposal VI was 85.1% and 13% for diabetic and non-diabetic subjects respectively.

Table 3.10: Diagnostic Efficacy for Proposal I – VII.

	CONTROLS				DIABETICS		
Proposal	**UAC**	**Sens%**	**Spec.%**	**NPV%**	**Sens.%**	**Spec.%**	**PPV%**
I	10.0	95.4	40.0	92.3	97.6	0.0	89.1
II	15.0	91.3	72.4	91.3	97.6	40.0	93.2
III	20.0	72.7	80.0	80.0	97.6	66.7	95.2
IV	25.0	45.4	83.3	67.5	97.6	100.0	100.0*
V	30.0	42.3	90.0	69.0	85.7	100.0	100.0
VI	25.0	66.7	87.0	95.2	100.0	85.7	97.5**
VII	30.0	44.4	81.4	87.2	97.2	70.0	92.3

*PPV, NPV; positive and negative predictive values *prevalence 89%, ** prevalence 85.1%*

The pre-test odds, likelihood ratio and the post-test odds for a positive test within the diabetic subjects were 5.71, 7.7 and 43.9 respectively. The posterior probability in obtaining a positive test from the diabetic subjects providing a single 2hr urine sample was 97.8% which is in good agreement with the positive predictive value of 97.5%.

3.3: OUTPATIENT DIABETIC POPULATION.

Two hundred and twelve (212) outpatient diabetic subjects most of them on oral hypoglycemic drugs, with 15 on insulin injection, were invited to participate in the study. They provided a 12-hr overnight urine collection after 2 previous random void urine samples tested albustix negative. Urine samples were brought to the clinic the morning after. Twenty-one percent (45) of the diabetics were males. Figure 3.4 shows the mean AER with age ranges; the peak mean AER was 28.6 μg/min for the ages 26-35years. They were all within the range of 15.0 –30.0μg/min. Figure 3.5 is the frequency distribution of AER in outpatient diabetics subjects. Sixty-one percent (132) of the outpatient diabetic subjects were excreting albumin below 20.0 μg/min and 37.7% (80) excreting albumin within the microalbuminuric range (20-200μg/min). Ninety-four percent (94%) of microalbuminuric subjects were excreting albumin between 20-100.0 μg/min. There was no correlation between age and AER.

Table 3.11 is the contingency table for the outpatient diabetic subjects using proposal VI for evaluating the usefulness of AB 580 for the screening of microalbuminuria where the sensitivity was 100% and specificity was 87%. The prevalence or prior probability of microalbuminuria in the diabetic population providing a single 12-hr overnight urine was 37.7%. The positive and negative predictive values were 82.5% and 100% respectively with 19 false positive cases.

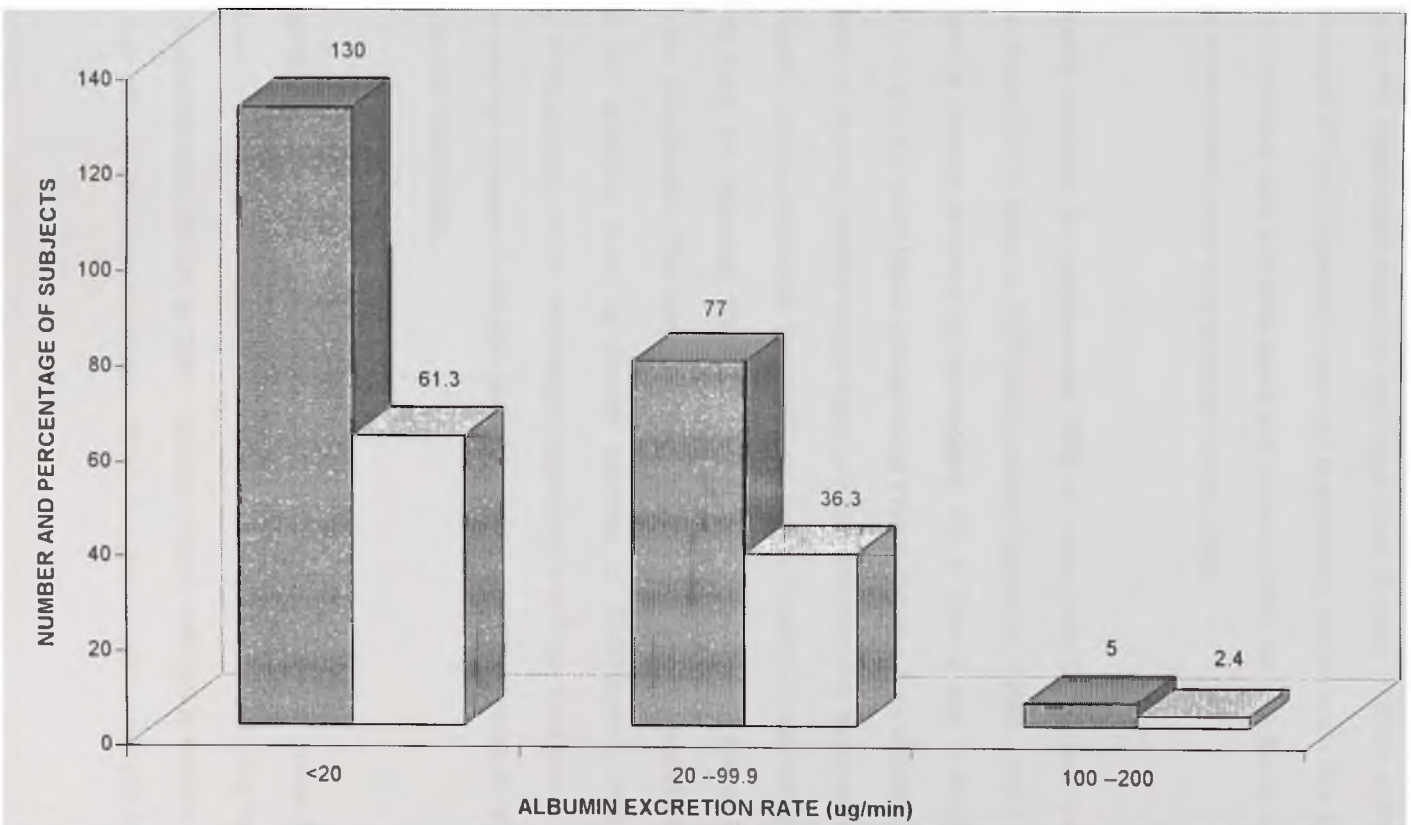
The pre-test odds, Likelihood ratio and post-test odds were 0.605, 7.4 and 4.48 respectively and a posterior probability of 81.7% to detect microalbuminuria, implying that 4 out of 5 subjects with increased N-AER were truly identified using the AB 580 assay. Twenty percent of positive tests will require additional urine collection to confirm the diagnosis of microalbuminuria. Another way of assessing the AB 580 is the diagnostic accuracy or efficiency i.e. the percentage of the sum of true positives and true negatives divided by population size. In the case of this study it was 92%.

Table 3.11: A Contingency Table for Screening Outpatient Diabetic Subjects Providing a Single 12 hour Overnight Urine Collection.

UAC (mg/L)	No. Of diabetic subjects N-AER(µg/min)		TOTAL
	≥20.0	<20.0	
≥25.0	80	19	**99**
<25.0	0	113	**113**
TOTAL	**80**	**132**	**212**

UAC - Urine albumin concentration; N-AER Overnight Albumin Excretion Rate

FIG. 3.3: FREQUENCY DISTRIBUTION OF 12HR AER IN KNOWN DIABETIC SUBJECTS



CHAPTER FOUR

4.0: DISCUSSION

Diabetes mellitus is the commonest cause of end stage renal disease (ESRD) in the U.S and Europe. The prevalence of Type 2 diabetes in particular is increasing, diabetics are now living longer because of improved medical care and more cases with diabetic ESRD are now being accepted for dialysis programs where formerly they were excluded *(Alzaid, 1996)*.

Diabetic nephropathy accounts for approximately 35% of cases with ESRD and the costs of treatment were in excess of $2 billion in 1991 (*ADA–position statement, 1998*). About 20-30% of Type 1 or 2 diabetics develop evidence of nephropathy, but in Type 2 only a smaller fraction progress to ESRD. Due to the much higher prevalence of Type 2 diabetes, they constitute over 50% of diabetics currently on dialysis. Several studies have now demonstrated that the onset and course of diabetic nephropathy can be ameliorated to a significant degree through several interventions, but such interventions have the maximum effect if it is instituted very early in the course of the development of the complication. The earliest clinical evidence of diabetic nephropathy is the detection of low but abnormal levels of albumin excretion of 20-200μg/min referred to as microalbuminuria. In the absence of any intervention, about 60-80% of Type 1 diabetics who develop microalbuminuria have an increase in albumin excretion rate of 10-20% annually to the stage of clinical albuminuria over 10-20 years.

Once clinical nephropathy occurs and in the absence of any interventions, the glomerular filtration rate (GFR) declines over a period of years at a variable rate varying from individual to individual. ESRD develops in approximately 35-50% of Type 1 diabetic subjects with clinical nephropathy within 10 years and in over 65% by 20 years *(Mathieson, 1993).* A higher proportion of Type 2 individuals

are found to have microalbuminuria and clinical albuminuria at diagnosis *(Uusitupa et al, 1987; Mykkänen et al, 1994)* and shortly thereafter. Type 2 diabetes is asymptomatic many years before diagnosis is made and also, the finding of albuminuria may be less specific for the presence of diabetic nephropathy as shown from biopsy studies *(Ruggenti and Remuzzi, 1998).*

Twenty to forty percent (20-40%) of Type 2 diabetics with microalbuminuria without intervention may progress to overt nephropathy, but by 20 years after diagnosis only 20% will have progressed to ESRD. The GFR also declines, varying between individual patients. However, there is a greater risk of dying from associated cardiovascular disease in older persons with Type 2 diabetes, which may therefore prevent the progression of incipient nephropathy to ESRD.

Microalbuminuria being an early manifestation of incipient diabetic nephropathy is also a marker of increased cardiovascular morbidity and mortality for both types of diabetes *(Mogensen and Christensen, 1984; Gosling, 1995a; Yudkin et al, 1988).* Thus the presence of microalbuminuria is an indication for screening renal disease, possible vascular disease and dyslipoproteinaemia *(Wincour, 1992)* and the institution of an aggressive intervention program to maintain non-progression, reverse or delay the progress of incipient diabetic nephropathy to clinical nephropathy and later ESRD (*Mogensen et al, 1995).*

In Ghana, tests for detecting microalbuminuria are non-existent, urinalysis is not done routinely for diabetic care in the country, and they are requested on account of suspected urinary tract infection. The possible reasons for failure to begin screening for microalbumin may be due to costs of reagent, kits, equipment, safety and lack of personnel with interest in diabetic nephropathy. The goal of a screening test is to be inexpensive, easy to perform, accurate, specific, and robust to minimize the number of false positives so that patients with the condition are not excluded from more intensive

testing. This is best met in screening for microalbuminuria by using 2hr and a 12hr-urine collection for measuring AER.

Kessler and Wolfbeiss (1992) described a non-immunological assay for detecting low concentrations of albumin 2-200 mg/L using the Albumin Blue 580 (AB 580) fluorescent probe with comparable detection limits and specificity to that of immunochemical methods and can be performed both manually and on automation. The advantages of using AB 580 are:

1. It is easy to perform without incubation or pre-treatment of urine samples,
2. The working range of 2-200mg/L covers the scale of microalbuminuria without dilution of urine samples,

(3) There is no need for antibodies,

(4) Reagent costs are low and no laboratory animals are needed for harvesting antibody production,

(5) Unlike immunochemical assay, the AB 580 assay does not report false negative values if the albumin concentration exceeds the upper limit of the working range,

(5) There is no Lot-to-Lot variation between AB 580 dye reagent and

(7) The reagent quality can be checked by measuring its absorbance at 580nm wavelength *(Kessler et al, 1997a).* The AB 580 dye is ideal for detecting microalbuminuria and potentially useful in measuring low concentrations of albumin.

To screen for microalbuminuria among Ghanaian diabetic subjects, the AB 580 dye must fulfill these requirements considering the economic status of Ghana. Microalbuminuria must exist as a sub-clinical disease. To screen for microalbuminuria, one need to find out if it exists undiagnosed in the general population and among diabetic subjects. Microalbuminuria must be well defined; its detection must improve the quality of life of patients through the institution of appropriate interventions, and if

the assay can be beneficial for service, research and management of individuals with the disease. Lastly, if its diagnosis may decrease the cost of managing diabetic renal disease in the country.

4.1 EVALUATION OF AB 580

The AB 580 assay is entirely new and for its acceptance as a diagnostic test it has to be evaluated for analytical precision and accuracy, positive and negative interferences, recovery and effect of storage. In this study the AB 580 assay was evaluated for precision, reproducibility, recovery studies and effect of storage on the sample. In the present study, the specificity, interference and accuracy tests were not done due to limited financial resources.

In this study the calibration curve of the AB 580 assay using human serum albumin as standard (1-200mg/L) was linear up to 150mg/L which covers the microalbuminuric range, but differs from what Kessler and colleagues reported (1–200.0mg/L). In one case from this study a urine albumin concentration of 198.1mg/L was obtained after dilution with calibrator diluents. This patient had albumin excretion rate beyond the microalbuminuric range but was albustix negative. The minimum detection limit was (3SD of blank) 1.4mg/L and 0.9mg/L for 2-200mg/L and 1-50mg/L range respectively.

The CV for 3 urine samples was less than 4.0% at (14.5mg/L, 50.2mg/L and 100mg/L. This study agrees with what was reported by *Kessler et al (1997b).* Triplicate assays were done on aliquots of urine samples with albumin concentrations of 6.0mg/L, 62.4mg/L and 130.2mg/L for 9 funs for reproducibility studies. The CV was less than 8.2%for all the 3 concentrations. For recovery, solutions with albumin concentrations 27.2, 56.0 and 100.0mg/L were added to 3 aliquots of urine. The recovery was between 95.6 and 120%, this finding compares favorably with what was published

by *Kessler et al (1997b)*. The results of this study suggest that the AB 580 assay is a reliable test for screening low quantities of albumin in urine.

The screening for microalbuminuria has become part of routine diabetes care in Europe and the US. This means that a larger number of patients will present urine samples for testing at any given period. This may overwhelm the service where there is lack of automation and especially in a developing country as Ghana where storage facility for urine samples is non-existent. On storage effects on urine albumin determination by the AB 580 assay in the present study, there were relative and absolute decreases in albumin concentrations at -20° and -80°C 30 days and beyond. This decrease in albumin content was not statistically significant at all the 3 storage temperatures and duration. The decrease in albumin concentration was non-uniform in the 26 samples. This is probably due to variations in rates of degradation that are found in urine. Elving et al, 1989 and Gomes et al, 1998 where the albumin content in frozen urine was underestimated, have confirmed this observation.

4.2: NON-COMMUNICABLE DISEASE SURVEY '99.

Microalbuminuria is reported to be more frequent among Type 2 diabetes, and it predicts increased morbidity and mortality due to cardiovascular causes *(Mogensen and Christensen, 1984)*. In the NCD-survey '99, 52 subjects who were non-diabetic and non-hypertensive served as the control group and 47 subjects who were newly diagnosed diabetics following oral glucose tolerance test formed the test group.

The prevalence of microalbuminuria (AER 20-200 μg/min) in the present work was 85.1% and 14% among the diabetic and the non-diabetic subjects respectively. The prevalence of hypertension was

diagnosis of Type 2 diabetes mellitus. The presence of hypertension at diagnosis of Type 2 is also commoner in individuals with incipient nephropathy (*Uusitupa et al, 1987; Dasmahapatra et al, 1994; Mykkänen et al, 1994*). *Nelson et al (1993a; 1993b; 1998)* found that elevated BP does occur in about 25-35% at the onset of Type 2 diabetes in Pima Indians. They concluded that elevated BP predicts microalbuminuria, and that raised arterial BP in Type 2 diabetes is not a consequence of nephropathy but may contribute towards its development. It predates the onset of Type 2 diabetes. The prevalence of microalbuminuria from this work during the survey was much higher than what Maiza *et al (1996) and Hemraj et al (1995)* reported from clinical cases in Mauritius and Algeria respectively, for Type 2 diabetes. The 85% prevalence of microalbuminuria in this study confirms that microalbuminuria is usually present in adult glucose intolerant subjects *(Nelson et al, 1998)*. *Hemraj et al (1998)* used Micral Test, a semi-quantitative test dipstick method that has a cut off value for UAC as 20 mg/L for detecting increased AER and Maiza et al *(1996)* used immunoturbidimetry in their work. The diabetic group was much older than the non-diabetic group (47.3 ± 9.0) vrs (38.7 ± 8.6 years) $p<0.05$.

The prevalence of arterial hypertension approximately doubles that of the non-diabetic population, and that diabetes is associated with increased cardiovascular risks and acceleration of morbidity and mortality in these patients *(JNC-V/ADA'1993)*. Several studies have found an association between elevated BP with progression of nephropathy and retinopathy. *Dasmahapatra et al (1994)* reported the prevalence of hypertension in African-Americans with Type 2 diabetes as 36-63%. The prevalence of hypertension from the present study from the non-communicable disease survey was 55.3% for the diabetic subjects. Using the *JNC–ADA* classification for hypertension, the prevalence increases among diabetic subjects with normo-, micro- and macroalbuminuria as compared to the *WHO* classification where the prevalence of hypertension is reported to be 12 – 25%.

In this study there was a significant difference ($p < 0.05$) for the mean BP between the non-diabetic and diabetic subjects especially with systolic BP. This is a well-known risk factor in Type 2 diabetes. However, *Mitchell et al (1997)* did emphasize that this relationship is more pronounced with nocturnal systolic BP From this work the normotensive diabetics were excreting more albumin in their urine than the non-diabetic subjects with a statistically significant difference. This observation means that the diabetic state may also play a role in the increased AER. However, hypertension seems to be an important determinant of AER, as AER is reduced on anti-hypertensive therapy in diabetics with nephropathy. It is not surprising that AER > 30 μg/min is predictive of early death from cardiovascular causes (*Jarret et al, 1984; Mogensen, 1984*). Between the normotensive and hypertensive diabetic subjects in this study, the AER was significantly higher in the latter (35.2 ± 5.37 vrs 56.5 ± 10.6μg/min); $p< 0.01$. The association of hypertension with microalbuminuria in Type 1 is usually found in cases with clinical nephropathy. Some studies have reported an association between the two in Type 2 diabetes, but its interpretation is rather difficult due to the cross-sectional nature of data and the fact that hypertension accompanies overt nephropathy

In the present study, the prevalence of 55.3% hypertension was much higher than what *Teuscher et al (1989) and Nelson et al (1993b)* reported. This difference may arise from the method of BP measurement and classification of hypertension *(WHO vrs JNC-V/ADA Classification).* Several researchers have reported a consistent association between elevated BP and microalbuminuria. This rise also falls within accepted normal range. On the average Type 1 microalbuminuric patients have BP levels that are approximately 10mmHg higher for age, sex and duration matched Type 1 diabetic subjects with normoalbuminuria, and that further changes in AER is related to changes in BP

4.2.1 Diagnostic Efficacy of AB 580 Assay From NCDS '99

For the diagnosis of microalbuminuria urine samples that are used in measuring AER require timed overnight (N-AER), 24 hour or short daytime collections (2-5) hours at rest. All these collections are cumbersome and tedious for patients and laboratory staff. Therefore a test or collection suitable for large scale screening of patients at regular intervals is required to preclude timed urine collection and that positive cases on screening will require confirmatory tests using timed urine collections. The FMU and 2-hour urine collection have several advantages; such as smaller sample volume, easily transportable, little or no timing required, sample can be obtained at the clinic and ensures subjects compliance *(Rowe et al, 1990).* The FMU sample is being used consistently for screening microalbuminuria with timed urine collection restricted to only positive cases from the screening process. On using un-timed urine collection, the urinary albumin concentration (UAC) is measured alone or corrected for urine flow by measuring urinary creatinine concentration as ACR. The UAC and ACR in the FMU has a better correlation with the overnight and 24 hour urine collections *(Gatling et al, 1985; Marshal and Alberti, 1986; Hutchinson et al, 1991; Marshall, 1991; Schwab et al; 1992*). It is suggested that UAC and ACR in random void urine are not suitable for screening microalbuminuria due to the dramatic effect of physical activity on AER in diabetic subjects *(Mogensen, 1971; Jefferson et al, 1985*).

In screening for microalbuminuria, various cut-off levels are deliberately chosen to give a maximum sensitivity with the belief that it will produce less false negative test results as possible, and also a negative test result should have a high negative predictive value. When a lower cut off value is used it will generate more false positive test results, which will require more subjects being followed up with confirmatory tests. Seven proposals were made using UAC cut-off values in predicting N-AER $\geq$ 15.0 and 20.0 μg/min among diabetics. Proposal VI had UAC $\geq$ 25 mg/L in a 2-hour daytime urine

collection predicting N-AER ≥ 20.0 μg/min. The sensitivity was 100% among diabetics and a specificity of 87% among non-diabetic subjects with the NPV as 95.2% from the survey. This compares with what *Marshall (1991)* reported, using different cut off values of ACR in a FMU to screen for microalbuminuria. The sensitivity was 88-100% and the specificity was 81-100%. The posterior or post-test probability was 97.8% for the diabetic subject.

4.3: OUTPATIENT DIABETIC POPULATION.

The AB 580 assay was used in an unselected number of diabetic subjects attending the Diabetes Management Centre at Korle Bu Teaching Hospital. Two hundred and twelve (212) patients had Type 2 diabetes, 15 subjects were on insulin therapy due to poor control. The male to female ratio was 1:4 mean age (range) 56.7 (15-80 years). Eighty (37.7%) were excreting N-AER ≥ 20.0 μg/min. On applying the diagnostic tests from proposal VI to the outpatient diabetic population with microalbuminuria, the prevalence was 37.7% with a NPV and PPV of 100% and 85.6% respectively. This implies that for UAC ≥ 25.0 mg/L, 4 out of 5 positive cases were truly identified as having AER ≥ 20.0μg/min with 1 case being misclassified. To detect UAC of 25.0 mg/L in this population using this assay, it will require a minimum detection limit of 10 mg/L for 2,500mL of overnight urine collection. This was the maximum urine volume collected during the study. However, the AB 580 had a minimum detection limit of 1.4 mg/L that will detect 3.5mg albumin in the same volume of urine collected. Therefore for a test with a minimum detection limit of 20mg/L such as Micral Test will misclassify most diabetics as normoalbuminuric as reported by *Kuori et al (1991).* From their study they did not recommend UAC alone as a screening test for microalbuminuria especially when using reagent tablets. *Hutchinson et al (1988)* reported that UAC ≥ 17.0mg/L in FMU was useful for screening "slight albuminuria". The AB 580 assay must be applied in future studies using FMU to ascertain its

diagnostic efficacy in predicting N-AER ≥ 20.0 ug/min instead of a 2-hr daytime collection as used in this work.

The usefulness of a new test for screening and diagnostic purposes depends on the following: the prevalence of the disease, sensitivity, specificity and predictive values of the test as applied to a general population. The prevalence of a disease is interpreted as the probability before the test is carried out (prior probability) that the subjects have the disease. The positive and negative predictive values are estimates of the same probability for those subjects who are positive and negative on testing i.e. posterior probability. The difference between the prior and posterior probabilities is one way of assessing the usefulness of the test *(Altman and Bland, 1994b).* One other way to assess the usefulness of the test is the Likelihood ratio. It implies that for a given test result, the comparison of the probability of obtaining a positive result if the patient is truly positive with the corresponding probability if the patient is healthy. The Likelihood ratio, which is the ratio of the true positive rate to the false positive rate (1-Sp), indicates the value of increasing the certainty about a positive diagnosis. The true and false positive rates can be used to plot a Receiver Operator Characteristic (ROC) curve with a prevalence of 37.7%. Diagnostic Accuracy or Efficiency is another way of assessing the reliability of a screening test. For a diagnostic sensitivity of 100% and diagnostic specificity of 82.5% respectively for AB 580 assay, the pre-test odds was 0.605, the post-test odds for 212 diabetic subjects was 4.68 and the Likelihood ratio was 7.69 and a posterior probability was 81.7%. The high Likelihood ratio and /or efficiency of 92% makes the assay valid and reliable to detect 25mg/L albumin in a 2 hour daytime collection predicting N-AER ≥ 20.0 ug/min in Ghanaian diabetics subjects.

4.4 CONCLUSION:

Microalbuminuria has been established as a strong marker of endothelial cell dysfunction in both diabetics and non-diabetics with cardiovascular disease. It predicts early nephropathy and cardiovascular events in diabetes. The prevalence of microalbuminuria for the newly diagnosed diabetic and non-diabetic, non-hypertensive adult Ghanaian population was 85% and 14% respectively confirming that microalbuminuria exists undiagnosed in the community. This observation suggests that microalbuminuria may be a feature of a pre-diabetic state and that an increase in AER may be related to an increase in glucose concentration and elevated BP Several reports have shown that microalbuminuria is present early in the course of Type 2 diabetes mellitus. *Mykkänen et al (1994)* reported that cases with impaired glucose tolerance and non-diabetic subjects with a parental history of diabetes and who are at risk for developing Type 2 diabetes had excess prevalence of microalbuminuria compared to non-diabetic control subjects.

Microalbuminuria in newly diagnosed Type 2 and in non-diabetic subjects with hypertension is probably of glomerular origin, possibly due to a localized increased pressure in the glomerular vessels that results in an increased AER. From the NCDS '99, non-diabetic subjects with microalbuminuria had higher BP levels than other subjects without microalbuminuria. Perhaps the association between microalbuminuria and a pre-diabetic state could result in elevation of BP. In this study old age and hypertension are noted risk factors for elevated AER in both diabetic and non-diabetic subjects.

The AB 580 assay is robust, does not require pre-treatment of urine, has a high diagnostic sensitivity, safe, inexpensive and results are reproducible for detecting microalbuminuria. Storage of urine at -20°C and -80°C beyond 30 days underestimated urine albumin concentration and will therefore

misclassify patients with borderline microalbuminuria; this underestimation was not statistically significant from this study.

The diagnostic efficacy of AB 580 in detecting and excluding microalbuminuria in the community was 100% sensitivity, 87% specificity, using a 2-hour daytime collection with UAC ≥ 25mg/L in predicting N-AER ≥ 20.0 μg/min, with a 97.5% PPV. The prevalence of microalbuminuria in the outpatient diabetic population was 37.7% with a diagnostic sensitivity, specificity and NPV of 100%, 82.5% and100%respectively. The AB 580 assay has a high post-test odds and Likelihood ratio of 4.68 and 7.69 respectively and a posterior probability of 82.4% making the assay useful, and valid for clinical, epidemiological and public health purposes. One limitation of this study is that the urine was not cultured to detect bacterial infections. However in this study urine samples were all leukocyte and nitrite negative on urinalysis. Besides this, elderly diabetic subjects are prone to urinary tract infection (UTI) than non-diabetic subjects. Thus inclusion of UTI cases is likely to add to the random variation towards the "null-hypothesis" i.e. finding risk differences.

Microalbuminuria was present at diagnosis of diabetes mellitus. Among the non-diabetic subjects microalbuminuria was associated with elevated BP. Thirty-seven percent (37.7%) of known outpatient diabetic subjects had microalbuminuria on providing a single 12-hr overnight urine collection. The role of microalbuminiura in the non-diabetic state needs to be explored further. Finally, microalbuminuria should be seen as an indicator of renal disease and the need to initiate treatment with regular monitoring. It is an important diagnostic tool that has to be standardized for age, sex, ethnicity, duration and type of urine collection. This study supports the use of a 2-hour daytime collection for screening microalbuminuria in Ghana, especially in a large population where the prevalence of diabetes is high or in a busy clinical setting. The Albumin 580 assay is highly sensitive, non-immunological, non-isotopic, reliable, robust and a safe method for screening microalbuminuria. The

AB 580 assay is recommended for routine assessment of microalbuminuria and for research into microvascular complications in Ghanaian diabetic subjects.

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APPENDIX.

1. PREPARATION OF SOLUTIONS.

A. Dye Stock (71 μmol/L AB 580 in isopropanol.)

10mg of AB 580 dye salt (Mr=306.8 was provided by Dr. Kessler and prepared as described by Kessler et al, (1997). 2.2mg of AB 580 was dissolved in isopropanol (Cat no. 4057; Sigma Chemical Co., St Louis, MO) to give dark blue solution. To check the concentration of AB 580, 1.0mL aliquot was diluted 10 fold with isopropanol. The Absorbance at 580nm of 1.0mL of diluted aliquot on a Spectronic 21 Spectrophotometer was $A_{580} = 1.0 \pm 0.05$ in a 1cm cuvette blanked against isopropanol then the crude stock is the dye stock.

B. Working buffer

The working buffer was constituted as follows; 3.0g of 3-N-Morpholinosulfonic free acid MOPS (Sigma M–1254), 9.0g of MOPS salt (Sigma M-9381), 12g sodium chloride, 1.0g EDTA (Sigma-4884) were dissolved in 900mL of distilled water and 100mL of isopropanol to give a pH of 7.4 ± 0.2

C. Working Dye solution

The working reagent is prepared fresh for each assay batch of 40 samples. 4.0mL of solution **A** is added to 100mL of solution **B**.

D. Calibrator diluent

2.7g KH_2PO_4, 0.9g K_2HPO_4 (BDH–Analar Grade Poole Chemical Ltd), 50mg Human immunoglobulin (IgG) fat free (Sigma G–4386), 0.5g of EDTA and 4.5g sodium chloride were dissolved in 500mL of distilled water to give a pH of 6.0 ± 0.5.

E. Albumin stock solution 2.0g/L

Human serum albumin solution obtained from Randox Laboratories Ltd, UK. (Lot No. 129AB) 45.0g/L.The stock solution was prepared by pipetting 8.88mL of human serum albumin and diluted with calibrator diluent to 200mL in volumetric flask. From this stock solution, standard solutions were prepared into concentrations of 1.0, 10.0, 25.0, 50.0, 100.0, 125.0, 150.0 and 200.0g/L. (Tab1-A)

Tab 1-A Preparation of Standard Solutions from Albumin stock.

Conc. Of standard (mg/L)	Vol. of stock solution (mg/L)	Vol. of calibrator diluent (mL)	Total Volume (mL)
1.0	0.05	99.95	100
10.0	0.50	99.5	100
25.0	1.25	98.75	100
50.0	2.50	97.5	100
100.0	5.00	95.0	100
125.0	6.25	93.75	100
150.0	7.50	92.5	100
175.0	8.75	91.25	100
200.0	10.00	90.0	100

D. Calibrator diluent

2.7g KH_2PO_4, 0.9g K_2HPO_4 (BDH–Analar Grade Poole Chemical Ltd), 50mg Human immunoglobulin (IgG) fat free (Sigma G–4386), 0.5g of EDTA and 4.5g sodium chloride were dissolved in 500mL of distilled water to give a pH of 6.0 ± 0.5.

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10.0	0.50	99.5	100
25.0	1.25	98.75	100
50.0	2.50	97.5	100
100.0	5.00	95.0	100
125.0	6.25	93.75	100
150.0	7.50	92.5	100
175.0	8.75	91.25	100
200.0	10.00	90.0	100