CHAPTER 1

LITERATURE REVIEW

1.1 AN OVERVIEW OF DIABETES

Diabetes is a disease state characterised by inadequate carbohydrate, lipid and protein metabolism, resulting from a relative or absolute deficiency of insulin. The inability to properly utilise these nutrients results in hyperglycaemia. The aetiology of the disease state is multifactorial. Depending on the specific type of diabetes, it includes genetic predisposition (Pociot & McDermott 2002; Gloyn *et al.* 2004; Qi *et al.* 2009), environmental factors such as infectious agents (Åkerblom *et al,* 2002; Roivainen 2006; Dotta *et al.* 2007), chemicals and nutrients (Turyk *et al.* 2009) and cell-mediated auto-immune destruction of β-cells of the pancreas (American Diabetes Association (ADA) 2005; Tang *et al.* 2008).

The disease has been classified into four main types: type 1 diabetes, type 2 diabetes, gestational diabetes and other specific types (Colagiuri 2006). In this study, all of the patients had type 1 diabetes. Microvascular complications associated with type 1 diabetes, namely diabetic nephropathy (DN), will form the focus of this literature review.

Type 1 diabetes usually presents in children and young adults and is characterised by the destruction of insulin-secreting β-cells of the islets of Langerhans (Rewers *et al.* 2004; Filippi & von Herrath 2007). Clinically, type 1 diabetes presents with symptoms of hyperglycaemia, including polyuria (the excretion of large amounts of water in excess of 2 liters/24 hrs) and polydipsia (excessive thirst).

Biochemical markers include auto-antibodies to islet cells (Hawa *et al.* 2000; Schiel & Muller 2000; Hoppu *et al.* 2006), glutamic acid decarboxylase, insulin and components of the insulin receptor, as well as altered frequency of polymorphisms occurring within immune-regulating genes in the human leucocyte antigen (HLA) region (Pozzilli & Di Mario 2001). Several cytokines, especially transforming growth factor (TGF-β) (Weston *et al.* 2003; Ziyadeh 2004) and tumour necrosis factor (TNF)-α (Alexandraki *et al.* 2006), contribute to the development of the disease.

Immunological and inflammatory mechanisms that identify the β-cells as foreign, mediate a cellular response leading to progressive destruction of the pancreatic cells and contribute to the development of the disease (Tuttle 2005; Mora & Navarro 2006; Williams & Nadler 2007; Fornoni *et al.* 2008).

Insulin deficiency results in the breakdown of tissue energy reserves. The major features of diabetes mellitus include inability to utilise glucose and overproduction of glucose leading to hyperglycaemia. Diminished protein synthesis and lipolysis result in hyperlipidaemia and weight loss. In the hyperglycemic state the renal threshold for glucose conservation is exceeded, resulting in osmotic diuresis which in turn results in polyuria, dehydration and thirst. In decompensated type 1 diabetes, free fatty acids liberated by unregulated lipolysis in adipose tissue, are converted to ketone bodies in hepatic mitochondria, including acetoacetate, acetone and beta-hydroxybutyrate. These ketone bodies dissociate to release hydrogen ions resulting in severe metabolic acidosis (diabetic ketoacidosis) which has a potentially fatal outcome (Underwood 2000). The administration of exogenous insulin is important for survival. Long-term exposure to elevated blood glucose levels, and the associated metabolic disturbances, leads to the development of complications including DN.

The Diabetes Prevention Trial of type 1 Diabetes (DPT-1) identified a group of patients with a different phenotype. Subjects were asymptomatic, had normal or impaired fasting glucose but had oral glucose tolerance tests (OGTT) that produced consistent 2-hour plasma glucose values greater than 11.1 mmol/l). These patients were still at risk of developing diabetic complications associated with raised plasma glucose (Greenbaum *et al.* 2001; Brownlee *et al.* 2006). Administration of exogeneous insulin to such individuals facilitates maintenance of normoglycaemia.

1.2 COMPLICATIONS ARISING FROM DIABETES

Diabetes mellitus and persistent hyperglycaemia over many years are known to promote cellular dysfunction and damage to small blood vessels and the greater vasculature, resulting in multiple organ failure (Keenan *et al.* 2007; Cade 2008; Tamura 2009). Heart disease, stroke, blindness, periodontal disease, central nervous system (CNS) disturbances and kidney dysfunction are some of the vascular complications arising from this metabolic disorder. Vascular complications arising from uncontrolled or poorly controlled diabetes are divided into two main groups, namely microvascular and macrovascular complications.

Microvascular complications include diabetic retinopathy (DR), diabetic neuropathy and diabetic nephropathy (DN) and usually occur in diabetic patients for a number of years before being diagnosed. Macrovascular complications include coronary artery disease (CAD), peripheral vascular disease (PVD) and cerebrovascular disease (CVD). These complications are discussed briefly below.

1.2.1 Microvascular Complications

In both type 1 and type 2 diabetes, it has been shown that the onset of microvascular complications arises from chronic hyperglycaemia (King *et al.* 2005; ADA 2010). Strict glycaemic control reduces and even delays the occurrence and rate of progression of end-organ disease. The main finding of the Diabetes Control and Complications Trial Research Group (DCCT, 1993) was that intensive therapy reduced the risk of developing retinopathy by 76% in those patients without retinopathy at outset. Furthermore, intensive therapy was shown to have a beneficial effect on reducing the risk of microalbuminuria and proteinuria by 39% and 54% respectively. There was also a 60% reduction in risk of clinical neuropathy (DCCT, 1993; UK Prospective Diabetes Study Group (UKPDS) 1998). Diabetic retinopathy, neuropathy and nephropathy are described below as examples of microvascular complication. Most emphasis is placed on DN since this forms the focus of the present study. The pathophysiology of DN is given below.

1.2.2 Diabetic Retinopathy (DR)

Diabetic retinopathy (DR) is a progressive disorder commonly found in diabetic patients. The disease progresses from a mild non-proliferative abnormality to a more severe non-proliferative form and finally to a proliferative DR. Non-proliferative retinopathy is characterised by changes to the blood vessels within the retina leading to bleeding, weakening of the blood vessels, fluid leakage and loss of circulation, without obscuring vision. In the untreated, the disease progresses to proliferative retinopathy. This occurs when blood vessels branch out in and around the retina, causing bleeding into the fluid-filled centre producing oedema and over time causing blindness (Fong *et al.* 2004).

The prolonged exposure of the microvessels in the eye to hyperglycaemia has been shown to cause basement membrane thickening, loss of pericytes (elongated contractile cells that wrap around endothelial cells of small vessels) and the development of microaneurisms (Hammes 2005). For patients with type 1 diabetes, marked DR usually develops within 20 years of diagnosis and most patients at the time of diagnosis have some form of DR (Kempen *et al.* 2004; Keenan *et al.* 2007). It is a leading cause of blindness in diabetic patients aged 20 to 74 years. In patients with type 1 diabetes the rate of disease progression is rapid (ADA 2004). Vahma (2005) and Wong *et al.* (2006) reported a higher incidence of DR in Africans compared to Caucasians.

Several pathological mechanisms have been proposed to play a role in the development of DR. These include increased flux through the aldose reductase pathway (Gabbay 2004), advance glycation end-product formation (AGEs) (Fong *et al.* 2004), stimulation of growth factors, increased reactive oxygen species generation (ROS) (Greenman *et al.* 2007) and stimulation of the local inflammatory response. Imbalances to these structural and metabolic processes leads to loss of cell function, restriction of blood flow and capillary blockages, resulting in tissue hypoxia and damage to the retina. The mechanism of involvement of these processes will be discussed later.

1.2.3 Diabetic Neuropathy

One of the most prevalent microvascular complications commonly seen in diabetic individuals is diabetic peripheral neuropathy (DPN), *viz* the presence of symptoms and/or signs of peripheral nerve dysfunction in patients with diabetes after the exclusion of other causes. Damage to both large myelinated as well as small thinly myelinated C fibres occurs (Vinik *et al.* 2001; Al-Sheklee *et al.* 2002). Damage to the autonomic system is referred to diabetic autonomic neuropathy (DAN).

Small fibre damage occurs initially in the lower limbs. Symptoms of numbness, pain and tingling, experienced in the hands and feet, carpal tunnel syndrome, slower rate of digestion and cardiovascular autonomic dysfunction, usually detected by an abnormal heart rate, are the earliest indicators of the onset of DPN (Lacomis 2002). A large proportion of diabetic patients have either a mild or severe form of nervous system damage. The loss of sensation experienced in the lower extremities and the development of ulceration, especially in the foot, is one of the leading causes of amputations in type 2 diabetes (ADA 2004).

The somatic nervous system form of DPN is commonly seen amongst diabetic patients. In some patients, effects on the autonomic nervous system may be encountered. Symptoms include sexual dysfunction, bladder incontinence and cardiovascular disease. Duration of hyperglycaemia, age, hypertension, smoking and dyslipidaemia are strong risk factors (Table 1) for the progression of DPN (King *et al.* 2005). Adults of short stature are associated with increased risk of microvascular complications in diabetes (Wade´n *et al.* 2009). Short adults are also at risk of adverse health outcomes related to diabetes, particularly CVD (Asao *et al.* 2006). Cardiovascular disease (CVD), metabolic ketoacidosis and microalbuminuria (MA) are strong predictors of DPN (Vinik 2002; Duby *et al.* 2004). The influence of blood pressure and lipids is discussed further in section 1.2.4.2.

Table 1. Risk factors associated with diabetic retinopathy (DR) and diabetic neuropathy (DN) adapted from the American Diabetes Association (ADA 2004).

Risk factors
• Hyperglycaemia
• Duration of diabetes
• Lipid and blood pressure
• Height
• Ethnicity

Adapted from the American Diabetes Association (ADA) positional statement 2004.

1.2.4 Diabetic Nephropathy (DN)

Diabetic nephropathy is discussed in some detail since it is a focus of the present study.

1.2.4.1 Epidemiology

Worldwide, DN is a leading cause of end-stage renal disease (ESRD) (*i.e.* kidney failure requiring dialysis or transplantation) in patients with diabetes. In the United States and most Western societies, DN is a leading cause of chronic renal failure. Of all the cases of ESRD in the U.S.A , 30-40% are due to diabetes. Both type 1 and type 2 diabetes lead to ESRD. In patients with type 1 diabetes with overt nephropathy, about 50% develop ESRD within 10 years and greater than 75% by the 20th year. In 1997 the cost of treatment of diabetic patients in the United States of America (U.S.A). with ESRD was in excess of $15.6 billion (ADA 2004).

In sub-Saharan Africa, hypertension and glomerular disease are the main causes of chronic kidney disease (CKD), affecting mainly young adults aged 20-50 years. In developed countries diabetes mellitus and hypertension are the main cause of CKD affecting mainly the middle-aged and elderly (Arogundade and Barsoum 2008). To date there are limited records of the prevalence of DN in South Africa. In a prospective study of clinical records of 219 patients with diabetes mellitus (DM) attending diabetic clinics in Durban, Motala *et al.* (2001) reported a 23.4% prevalence of persistent proteinuria (Blacks 25%, Indians 18.2%) and hypertension 34% (Blacks 41.7%, Indians 9.1%).

1.2.4.2 Pathogenesis of Diabetic Nephropathy

Diabetic nephropathy (DN) is a disease state involving the kidney, especially the nephron of diabetic patients. A hyperglycaemic environment promotes DN. Pathological changes to the structure and function of the kidney include hypertrophy, increased thickness of the glomerular basement membrane (GBM) and progressive accumulation of extracellular matrix proteins (ECM) in the mesangium and interstitium (Ziyadeh 2004). Nodular glomerulosclerosis, tubulo-atrophy and interstitial fibrosis are also characteristic of the disease (Alebiosu *et al.* 2002). Functional changes occurring in the nephron at the level of the glomerulus lead to glomerular hyper-filtration, subsequent proteinuria, systemic hypertension and loss of kidney function (Dronavalli *et al.* 2008). Clinical hypertension aggravates the condition, causing gradual loss of kidney function. Risk factors associated with DN are shown in Table 2.

Table 2: Risk factors associated with Diabetic Nephropathy

Risk factors
• Genetic susceptibility
• Hypertension
• Race
• Hyperglycaemia
• Age
• Dyslipidemia
• Male gender

Adapted from Ayodele *et al.* (2004)

Genetic as well hemodynamic factors play a role in disease progression. In type 1 diabetes, genetic predisposition may be the most important risk factor for nephropathy as only half of the patients with DN have poor glycaemic control while others develop this complication even with relatively good glycaemic control (Rich 2006). In patients with diabetes, elevated dyslipidemia (raised levels of triglycerides, low levels of high-density lipoprotein cholesterol, and high levels of low-density lipoprotein) contribute to the risk of CVD (Colhoun *et al.* 2004;
Kumar & Singh 2010). Furthermore, men have increased risk for the development of DN compared to women (Ayodele *et al.* 2004; Coresh *et al.* 2005).

A number of metabolic disturbances are associated with DN. Increased plasma glucose levels stimulate the renin-angiotensin (RAS) system which in turn causes an increase in renal glomerular pressure. Angiotensin II (Ang II) stimulates overproduction of collagen IV in glomerular cells contributing to ECM and GMB thickening via activation of TGF-β and vascular endothelial growth factor (VEGF) (Iglesius-de la Cruz *et al.* 2002; Chen *et al.* 2004). Glomerular cells exposed to repeated cycles of stretch and relaxation (as a result of increased glomerular filtration or glomerular pressure) have been shown to alter their structure. Podocytes exposed to a diabetic milieu have been shown to alter their orientation in response to stretch/relaxation, resulting in increased cell loss (Endlich & Endlich. 2006). The activation of TGF-β under conditions of cycles of stretch/relaxation (Durvasula *et al.* 2004) has been shown to increase podocyte apoptosis (Durvasula *et al.* 2005).

1.2.4.3 Criteria for early diagnosis of DN

According to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF/KQOQI) clinical practice guidelines (2007), the following screening criteria are used to evaluate patients with diabetes and diagnose CKD and early DKD. Initial screening should commence 5 years after the diagnosis of type 1 diabetes; (A) or from diagnosis of type 2 diabetes. (B)

Screening should include:

- Measurements of urinary albumin-creatinine ratio (ACR) in a spot urine sample; (B)
- Measurement of serum creatinine and estimation of GFR.(B)

An elevated ACR should be confirmed in the absence of urinary tract infection with 2 additional first-void specimens collected over the next 3 to 6 months. (B)

- Microalbuminuria is defined as an ACR between 30-300 mg/g.

Macroalbuminuria is defined as an ACR > 300 mg/g.

● 2 of 3 samples should fall within the microalbuminuric or macroalbuminuric range to confirm classification.

In most patients with diabetes, CKD should be attributable to diabetes if:

● Macroalbuminuria is present; (B) or

● Microalbuminuria is present in the presence of diabetic retinopathy, (B) in type 1 diabetes of at least 10 years duration. (A)

The above criteria is commonly used for the diagnosis of DN. Studies in Japan have shown that raised urinary type IV collagen can be used to identify those diabetic patients at risk of diabetic renal disease. The Diabetic Nephropathy Committee in Japan recommends the use of revised criteria for the early diagnosis of DN (Inomat *et al.* 2005). These include:

1. Urinary albumin measured by immunoassay using a morning spot urine sample in diabetic patients without proteinuria or with dipstick-positive (+1) proteinuria.
2. A urinary albumin-to-creatinine ratio ranging from 30 to 299 mg/g Cr in 2 or more of 3 specimens may be diagnosed as microalbuminuria.
3. Two alternatives *i.e.* the urinary albumin excretion rate of 30-299 mg/24hr urine collection or 20-199 microgram/min in timed urine collection, can be used to detect microalbuminuria.
4. Renal hypertrophy and elevated urinary type IV collagen may indicate the existence of diabetic renal disease.
5. Microalbuminuria originating in non-diabetic diseases should be excluded.

1.2.4.4 Microalbuminuria (MA) as a marker of DN

The earliest marker of DN is the detection of small amounts of protein in the urine. This is referred to as MA. There are currently three methods used for screening:

1. Albumin-to-creatinine ratio in a random spot collection.
2. 24-h collection with creatinine, simultaneous measurement of creatinine clearance.
3. timed collection (4-hours or overnight).

Microalbuminuria is established if urinary albumin excretion is ≥ 30mg/24 h (equivalent to 20 µg/min on a timed specimen or 30 mg/g creatinine on a random sample (collection on at least two occasions within a 3 to 6 month period). If left unchecked, approximately 80% of patients with type 1 diabetes progress to overt nephropathy (*i.e.* urinary albumin excretion > 300 mg/24 hours, > 200 mg/min in a timed specimen or > 300 µg/mg creatinine in a spot urine) within a period of 10 to 15 years. In type 2 patients approximately 20% to 40% progress within 15 years of the appearance of MA (Remuzzi *et al.* 2002; Eisenbarth 2004; ADA 2004).

Ultrastructural changes to the BM are responsible for the increased permeability and loss of albumin during filtration across the membrane. Functional changes in the GBM selective permeability both in animal models (Jeansson *et al.* 2006) as well as in humans (Dalla *et al.* 2003; Karumanchi *et al.* 2005) indicate that loss of both size and charge selectivity are associated with microalbuminuria in diabetes.

Since the introduction of MA as a screening tool for incipient nephropathy in diabetic patients, it has become clear that a significant proportion of subjects with normoalbuminuria progress to overt nephropathy without passing through the microalbuminuric phase (Caramori *et al.* 2000). Proteinuria and MA have been shown in a 5-year prospective study to be independently linked to cardiovascular risk and death (Romundstad *et al.* 2003). This has been further supported by recent studies in diabetic individuals (UKPDS 1998; Donnelly *et al.* 2003; Ochodnicky *et al.* 2006).

Microalbuminuria together with hypertension is an important risk factor for the development of cardiovascular disease and renal failure. The presence of MA lacks specificity as a sensitive marker in DN. To most clinicians, the reporting of microalbuminuria and/or proteinuria sparks the clinical diagnosis of DN and hints of the incipient renal failure typically seen in diabetic patients. The absence of MA and/or proteinuria falsely reassures clinicians that no pathology may be present and often patients are not investigated (Cohen *et al.* 2005). Numerous researchers have recently brought to light the fact that normoalbuminuric type 1 and type 2 diabetic patients may have reduced GFR in the absence of MA (Caramori *et al.* 2000; Caramori *et al.* 2003; Garg *et al.* 2002; Amin *et al.* 2005; MacIsaac *et al.* 2006).

Furthermore, MA is found in the non-diabetic, normotensive population and is a marker of cardiovascular risk factors (Hillege *et al.* 2001; Atkins *et al.* 2004; Hari *et al.* 2010). Recent studies have shown that there is a higher incidence (50%) of remission (Perkins *et al.* 2003; Araki *et al.* 2005) compared to 15 to 25% progressing to proteinuria (Roy *et al.* 2007; Amin *et al.* 2008). Thus a reliable indicator of early stages of renal pathology is very important in the clinical management of DN, and alternatives to MA are always being sought.

It has been established that the use of certain drugs typically used to treat hypertension in renal disease, such as angiotensin converting enzyme inhibitors (ACE inhibitors) and angiotensin II (Ang II) receptor inhibitors, can delay progression of nephropathy, even in normotensive individuals (Caramori *et al.* 2000; Thomas & Atkins 2009). Angiotensin converting enzyme (ACE) inhibitors have been shown to decrease systemic blood pressure as well as reduce the glomerular pressure. Patients with blood pressure below 130 mm/Hg were shown to have a lower chance of developing MA compared to those whose blood pressure were above 130 mm/Hg. Decreasing intra-glomerular pressure is associated with a lower rate of decline of GFR. Lowering of overall blood pressure was shown to delay onset of MA and microvascular and macrovascular complications of diabetes (DCCT 1993; Ruggenenti *et al.* 2004).

1.2.5 Macrovascular Complications

Diabetes mellitus is known to contribute to the risk of CVD. Macrovascular complications include diseases of the coronary arteries, peripheral arteries and carotid vessels. These are highly prevalent in type 2 diabetes. Coronary artery disease (CAD) is associated with much of the morbidity and mortality in patients with diabetes (Romundstad *et al.* 2003; Mohan 2007).

Atherosclerosis is a common precursor to CAD. It is a complex and multifactorial pathological process characterised by a slow and progressive accumulation of lipids on the artery wall, accumulation of smooth muscle cells, and necrotic debris in the intima of the elastic arteries (Ashley *et al.* 2007; Mohan 2007). The deposition of fatty acids and inflammation at the site causes a progressive decrease in elasticity, leading to intimal medial thickening, plaque formation and finally clogging of the artery. Artherosclerosis in diabetics increases the risk of development of CAD, cereobrovascular disease and peripheral vascular disease (PVD) (Mohan 2007). Tissue loss and the onset of gangrene is a major feature of patients with advanced peripheral artery disease (PAD) and is responsible for many of the non-trauma associated amputations especially in diabetic patients (King *et al.* 2005; Singh *et al.* 2005). The macrovascular complication of DM are summarised in Table 3.

Table 3. Summary of chronic macrovascular complication of Diabetes

Chronic complications	**Clinical presentation**
Coronary artery disease (CAD)	Myocardial infarction and angina pectoris
Cerebrovascular disease (CVD)	Stroke and transient ischemic attacks
Peripheral arterial disease (PAD)	Pain, aches, loss of foot pulse etc.

1.3 BIOCHEMICAL PROCESSES UNDERLYING DEVELOPMENT OF COMPLICATIONS OF DIABETES MELLITUS

Many biochemical processes have been proposed to underlie the development of complications in diabetes. Brownlee (2001) hypothesized the integration of four biochemical mechanisms contributed to the development of microvascular complications. These were:

1. increased advanced glycation end-product (AGE) formation
2. increased polyol pathway flux
3. activation of protein kinase C (PKC) isoforms and
4. increased hexosamine pathway flux.

All four mechanisms had previously been recognised individually as consequences of chronic hyperglycaemia. Chronic hyperglycaemia has been shown to increase and cause disturbances to several metabolic pathways (polyol pathway, hexosamine pathway), initiate structural changes to proteins by increased production of advanced glycation end-products (AGEs) and increased production of protein kinase c (PKC) and other cell signaling mechanisms (King *et al.* 2005; Cade 2008). These abnormalities in the diabetic milieu promote the formation of reactive oxygen species (ROS) and initiate local inflammatory responses that are responsible for the micro-vessel damage in tissues and organs. These four mechanisms, and other biochemical processes proposed as major contributors to diabetic complications, are reviewed in the sections which follow.

1.3.1 Protein glycation

Proteins can react non-enzymatically with glucose. This reaction is referred to as the Maillard reaction or non-enzymatic glycation. It is dependent on the concentration of circulating glucose or other reducing sugars (*e.g.* fructose). Hence in a diabetic state, glycation occurs freely.
The first step of the Maillard reaction is Schiff base formation. Amino (N-terminal) or ε-amino groups on proteins react with the carbonyl groups of reducing sugars. Instability is characteristic of the Schiff base, which dissociates easily. Schiff bases rearrange over time to form more stable compounds called Amadori products (AP). Reactions involving fructose are referred to as Heyns products (Thorpe & Baynes 2003; Goldin *et al.* 2006). Figure 1 shows a summary of the steps involved in the formation of advanced glycation end products.

An equilibrium between Schiff bases and AP exists which is dependent on glucose concentration. Once AP have formed, the reaction can proceed even when glucose concentrations are normal. Over time, AP undergo complex reactions (*e.g.* metal-catalysed oxidative reactions), forming a range of permanent protein adducts, including cross-linked protein. Examples of AGEs include N-ε-(carboxymethyl lysine (CML) or N-ε-carboxyethyl lysine (CEL) and pyralline (Hein *et al.* 2003; Goh & Cooper 2008). Some AP over time form reactive dicarbonyl compounds (*e.g.* deoxglucosone, glyoxyl or methylglyoxyl). These compounds can bind to proteins and tissue and have been shown to alter protein structure and function by facilitating cross-linking (Tuttle *et al.* 2005; Koka *et al*. 2006).

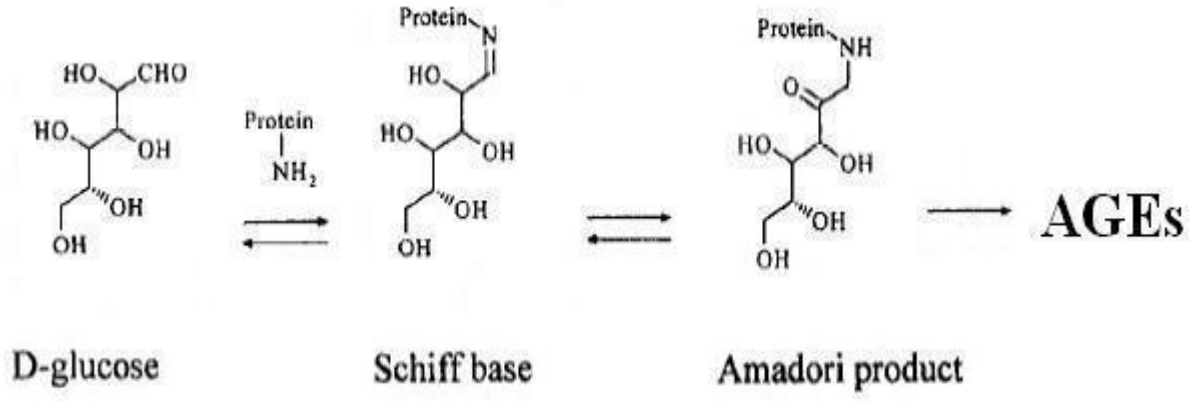

Figure 1 Formation of advanced glycosylation end products. Schematic representation of the formation of advanced glycosylation end products in a diabetic milieu. Adapted from Ulrich and Cerami. (2001). *Recent Progress in Hormone Research* 56:1-21.

The non-enzymatic glycation of proteins and the formation of AGEs is thought to be a pathological link to many of the complications observed in diabetes, including nephropathy and retinopathy (Brownlee 2001; Kowluru 2005; Kowluru & Chan 2007). These react with several receptors most notably the receptor for advanced glycation products (RAGE) (Gu *et al.* 2006; Paavonen *et al.* 2008), which may trigger intracellular metabolic events. In *in vitro* studies in glomerular and mesangial cells, glycated albumin (GA) and AGE-rich proteins have been shown to increase PKC activity leading to ECM expansion (Chen *et al.* 2001; Goldin *et al.* 2006). In type I diabetes, the increased circulation of AGEs has been shown to precede the development of microvascular complications (Katakami *et al.* 2005).
Gu *et al.* (2006) showed that AGEs, especially CML, induce expression of MCP-1 in podocytes, through RAGE activation which stimulates ROS followed by upregulation of interleukin-6 (IL-6) which triggers tubular atrophy, interstitial infiltrates and inflammation.

1.3.2 Increased Polyol Pathway Flux

Aldose reductase (AR) is the rate-limiting enzyme of the polyol pathway, which is the pathway by which glucose is metabolised to sorbitol and finally to fructose. It catalyses the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of a variety of carbonyl compounds, including glucose and galactose (Petrash 2004). The enzyme has a low affinity for glucose and under euglycaemic conditions, only a small percentage of glucose is metabolised via this pathway. Under hyperglycaemic conditions however, glucose is converted to sorbitol by the enzyme sorbitol dehydrogenase using nicotinamine adeninine dinucleotide (NAD^+) as a cofactor. Fructose is phosphorylated to fructose-3-phosphate and further metabolised to 3-deoxyglucosone (3-DG) that has been shown to be involved in AGE formation (Schalkwijk *et al.* 2004; Takeuchi *et al.* 2010).

Increased flux through the polyol pathway causes a decrease in NADPH levels and reduces the level of reduced gluthathione (GSH), which is a reactive oxygen scavenger. A number of mechanisms have been proposed to explain the potential detrimental effects of hyperglycaemia-induced increases in polyol pathway flux. These include sorbitol-induced osmotic stress, decreased (Na^+ + K^+) ATPase activity, an increase in cytosolic NADH/NAD^+ ratio and a decrease in cytosolic NADPH (Brownlee 2001, Chung *et al.* 2003).

Polyol pathway flux and AR activity have been investigated in various animal and human models. Increased AR activity was identified in Müller glia, vascular pericytes, retinal ganglion cells and in human cells, indicating a role for glucose metabolism via the sorbitol pathway (Dagher *et al.* 2004). Studies have identified hyperglycaemia as the stimulus for sorbitol/fructose-induced oxidative stress in animal models (Asnaghi *et al.* 2003; Obrosova *et al.* 2003). Studies by Obrosova *et al.* (2005) showed that cells treated with aldose reductase inhibitors (ARI) (fidarestat, 16 mg/kg/day) ameliorated nitrosative stress (a condition where the production of highly reactive nitrogen-containing chemicals *e.g.* nitrous oxide (NO) exceed the bodies ability to remove and eliminate them) and poly(ADP-ribose) polymerase activation (PARP) induced by hyperglycaemia. Gleissner *et al.* (2008) showed that high glucose concentration induced oxidative stress in 20% to 30% of macrophage foam cells due to increased AR activity. Cells exposed to oxidised low-density lipoprotein (oxLDL) in a hyperglycaemic environment also showed an increase in AR gene expression.

1.3.3 Activation of protein kinase C (PKC)

Protein kinase C (PKC) belongs to a group of serine/therionine kinases that catalyse the transfer of phosphate groups from adenosine triphosphate (ATP) to a target protein (enzymes, cell membrane receptors or ion transport channel) (Dempsey *et al.* 2000; Nishikawa *et al.* 2000). Twelve isoforms of the PKC family have been identified and these isoforms show varied tissue localisation, substrate affinity and function.

Hyperglycaemia has been shown to activate the production of PKC-β by the *de novo* synthesis of diacyl glycerol (DAG), an intracellular activator of PKC. Nine of the eleven isoforms of the PKC family are activated by DAG. Activation of PKC-β has been associated with basement membrane (BM) thickening, ECM accumulation and increased cell turnover and permeability (Way *et al.* 2001). Increased levels of DAG have been found in cultured microvascular cells, and in the retinas and renal glomeruli of diabetic animals (Rask-Madsen & King 2005).

Several other activators of PKC activity have been identified in the diabetic milieu. These include the binding of AGE receptors (Toth *et al.* 2008), increased reactive oxygen species (ROS) generation (Pricci *et al.* 2003), activation of various growth factors and increased synthesis of Ang II (Kang *et al.* 2006). Abnormal activation of PKC-β is implicated in the decreased glomerular production of nitric oxide (NO) induced by experimental diabetes and in smooth muscle cells in a state of hyperglycaemia (Rask-Madsen & King 2005).
Gallo *et al.* (2005), using human umbilical cells, showed that metformin (an anti-diabetic drug used mainly to treat patients with type 2 diabetes) was able to inhibit PKC-β-induced oxidative stress in a hyperglycaemic environment.

1.3.4 The Hexosamine Biosynthesis Pathway (HBP)

The hexosamine pathway is responsible for the synthesis of amino sugars. The substrate, fructose-6-phosphate, is converted to glucosamine-6-phosphate (GlucN-6-P). Glutamine serves as an amino donor for the rate-limiting enzyme, glutamine:fructose-6-phosphate-aminotransferase (GFAT). Glucosamine-6-phosphate is converted to uridine-5-diphosphate-N-acetylglucosamine (UDPGlucNAc) which is a precursor for the synthesis of proteoglycans, glycosaminoglycans, glycoprotein and glycolipids. Uridine-5-diphosphate-N-acetylglucosamine also serves as a substrate for the formation of O-linked glycoslyation using the enzyme O-GlcNAc transferase (Wells *et al.* 2001).

The hexosamine pathway has been implicated in the pathogenesis of diabetic complications (Brownlee 2001). Under hyperglycaemic conditions and increased glucosamine concentrations, increased levels of plasminogen activator inhibitor-1 (PAI-1) were mediated by transcription factor specificity protein 1 (Sp1) in vascular smooth muscle cells (Du *et al.* 2000; Buse 2006). Furthermore, TGF-β activity and mRNA levels were increased, stimulating increased fibronectin production following over-expression of GFAT. Inhibition of GFAT activity was shown to ameliorate these effects (Du *et al.* 2000; Weigert *et al.* 2003). Covalent modification of Sp1 by GlcNAc may explain the link between activation of the hexosamine pathway and the hyperglycaemia-induced changes in transcription of the gene for PAI-1 and PKC-β, as observed in glomerular mesangial cells (Goldberg *et al.* 2002; Goldberg *et al.* 2006).

1.3.5 Cytokines

Cytokines are a group of low molecular weight polypeptides possessing autocrine, paracrine and juxtacrine effects. These molecules have been placed into various classes including interleukins, tumour necrosis factors, interferons, colony stimulating factors, transforming growth factors and chemokines. Cytokines play a significant role in humoral-mediated immunity through complex and co-ordinated inflammatory responses (Williams & Nadler 2007).

Crook (2004) and Pickup (2004) have shown that chronic low-grade inflammatory processes contribute to the pathogenesis of diabetes mellitus. Inflammatory processes and cytokine activation have been linked to many of the microvascular complications in diabetes (Navarro & Mora 2005; Mocan *et al.* 2006; Navarro & Mora 2008). Growing evidence suggests that TGF-β plays a crucial role in the pathogenesis of diabetic nephropathy (Ziyadeh 2004; Pantsulaia 2006).

The TGF-β system is composed of multifunctional cell-cell signaling proteins that regulate cell proliferation and metabolism of ECM proteins (Koli *et al.* 2001). Enhanced local production of MCP-1 results in monocyte attraction to the site and enhances intracellular adhesion molecule-1 (ICAM-1) production. This significantly enhances leukocyte adhesion to mesangial cells (Giunti *et al.* 2006). Amplification of the inflammatory process contributes to glomerular injury and the release of ROS causing the destruction of podocytes and this contributes to the pathology of the glomerulus (Endlich & Endlich 2006).

1.3.6 Haemodynamic Factors

Under hyperglycaemic conditions, glomerular hyperfiltration occurs as a result of decreased resistance in afferent and efferent arterioles of the glomerulus. Haemodynamic factors (prostanoids, NO, vascular endothelial growth factor VEGF-A, TGF-β1 and Ang II) facilitate the loss of albumin through the glomerulus and contribute to ECM protein accumulation, injury to podocytes and to the effect of mechanical strain which eventually lead to their loss (Wolf *et al.* 2005; Wolf & Ziyadeh 2007; Ziyadeh & Wolf 2008).

The haemodynamic renin-angiotensin-aldosterone system (RAAS) has been shown to orchestrate many of the pathological micro- and macro-vascular complications observed in diabetes by affecting various tissue responses including vasoconstriction, inflammation, oxidative stress, cell hypertrophy and increased angiogenesis with resultant fibrosis (McFarlane & Sowers 2003; Rossing *et al.* 2006). Angiotensin II is a potent physiological effector of the RAAS.

The level of Ang II is upregulated in diabetes (Doublier *et al.* 2003; Awad *et al*. 2005;) and its interaction with angiotensin Type 1 receptor (AT1R) has been shown by numerous studies to promotes fibrosis by various processes. These include stimulating increased synthesis of the ECM (Thomas *et al.* 2000; Tikellis *et al.* 2008), promoting apoptosis (Ding *et al.* 2002), and enhancing the release of several inflammatory cytokines including vascular endothelial growth factor (VEGF), TGF-β1 (Ziyadeh 2004; Chen *et al.* 2005) and connective tissue growth factor (CTGF) (Liu *et al.* 2007).

1.3.7 Oxidative stress

Tissue hypoxia is a disruption of local metabolism as a result of imbalances in oxygen supply and consumption . Oxygen is important for metabolic processes including ATP formation by oxidative phosphorylation in the mitochondria. Under normal conditions, the primary function of molecular oxygen (O_2) in mitochondia is to serve as terminal electron acceptor in respiration. Some O_2 is reduced to ROS as an unavoidable side-effect. Under various disease states, including diabetes, ROS production is increased (Droge 2002; Singh *et al.* 2008). Excess production of ROS, occurring under conditions of endogeneous or exogeneous overproduction or inefficient removal, results in the development of oxidative stress.

Chronic oxidative stress results in the damage of macromolecules such as DNA, lipids and proteins, disrupts cellular processes and is responsible for several disease processes (Cutler 2005; Nangaku *et al.* 2008). In diabetes, sources of oxidative stress include auto-oxidation of glucose, redox balance shifts, imbalances in reduced gluthathione (GSH) and impaired superoxide dismutase (SOD) activity (Haskins *et al.* 2003; Kowluru *et al.* 2004).

The preceding sections have reviewed the biochemical mechanisms thought to contribute to the development of diabetic complications, especially DN, which is the focus of this study. To further support development of the study hypothesis, the following sections consider structure of the GBM, the site of much of the hyperglycaemia-induced alterations associated with DN. Common markers of glycaemic control and renal function used in the clinical management of DN are also described.

1.4 EXTRACELLULAR MATRIX AND BASEMENT MEMBRANE COMPONENTS

The ECM is the matrix of basement membranes including the GBM of the kidney. It functions both as scaffolding and as a support for the attachment of cells and organs. This complex network allows for the exchange of information between cells and facilitates several biochemical processes for cell development, migration, differentiation and repair (Hayden *et al.* 2005).

Basement membranes (BM) are composed of continuous sheets of specialised ECM material (50-100 nm, basal laminae) composed of collageneous and noncollageneous glycoproteins and proteoglycans. They are found whenever cells (except connective tissue cells) meet connective tissue. The main components of the BM include type IV collagen, fibronectin (FN), laminin, heparin sulphate proteoglycans (HPSG), nidogen or entactin, and osteonectin. Type IV collagen and laminin self-assemble into the main scaffolding and are crucial for maintaining structural stability (Pösch *et al.* 2003; McKee *et al*. 2009).

Nidogen/entactin and perlecan bridge the collagen IV/laminin superstructure and increase the stability of the structure. Several other components make up the BM, including agrin, fubulin, type XV collagen, type XVIII collagen, secreted protein acidic cystein-rich (SPARC), osteonectin, basement membrane 40 and 90 (BM40/BM90). The specific combination of these units contributes to the tissue specificity of the BM distributed throughout the body (LeBleu *et al.* 2007).

The GBM of the kidney additionally functions as a filter, producing a protein–free filtrate of blood plasma. The glomerular filter has selective properties based on charge and size. This restricts passage of macromolecules across the capillaries. The accumulation and cross-linking of the AGE-modified matrix proteins may have a significant effect on the ability of the glomerulus to sieve out larger proteins. Murdaugha *et al.* (2009) demonstrated that AGE-modified laminin exhibited decreased binding to type IV collagen and heparan sulfate proteoglycan. Albumin modified with AGEs has abnormal conformation compared to native albumin and thus filters more easily through the GBM than native albumin (Londono & Bendayan. 2005; Cohen *et al.* 2006).

Extracellular matrix (ECM) accumulation in the glomerular mesangium and tubulointerstitium are characteristic of DN. Imbalances in the synthesis and degradation of ECM components leads to the accumulation of collagens, fibronectins and laminins. The ECM proteins have a slow turnover and are therefore susceptible to the AGE modification which affects their structure and function (Forbes *et al.* 2003). Advanced glycation end-products have been shown to alter ECM composition causing increased expression of FN (Forbes *et al.* 2003), collagen I and IV (Abe *et al.* 2004; Pozzi *et al.* 2009) both in the presence and absence of glycaemia. Lam *et al.* (2004) showed that increased ECM production is accompanied by increased numbers of interstitial fibroblasts, myofibroblasts and infiltrating macrophages in DN. Figure 2 shows the structure of the kidney and the location of the GBM.

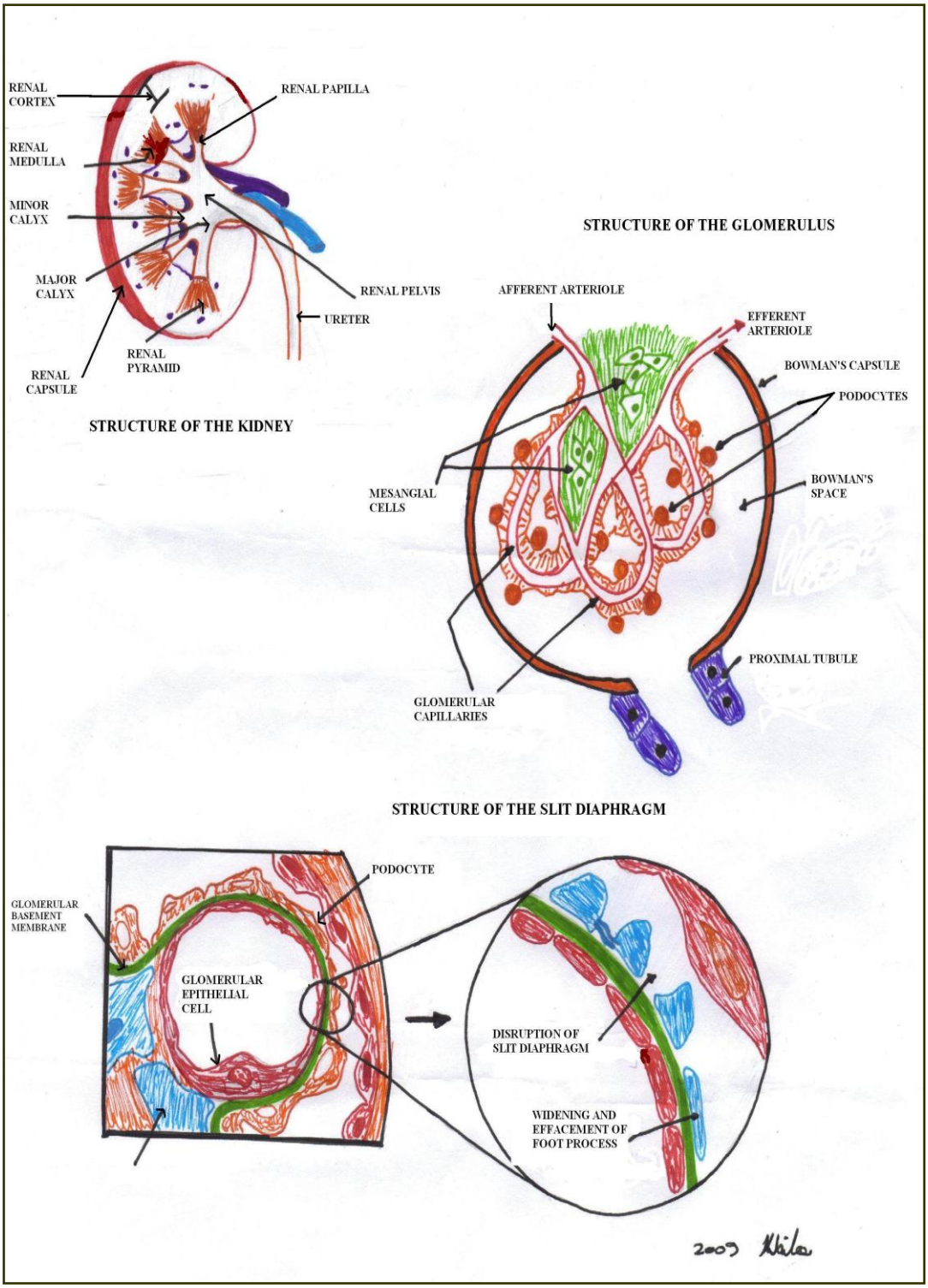

Figure 2. Schematic diagram of the glomerulus indicating the slit diaphragm and widening of the foot processes. Adapted from Jefferson *et al.* (2008).

In diabetes, type IV collagen (Yoshioka *et al*, 2004; Pozzi *et al*, 2009), FN (Yung *et al.* 2006; Wang *et al.* 2008) and laminin (Moriya *et al.* 2001), which are normal constituents of the GBM, are increased in the kidney. Decreased levels of proteoglycans have been found in the diabetic kidney in mesangial matrix and renal tubular cells, and have been shown to play a role in the development of diabetic complications (Schaefer *et al.* 2001; Rienstra *et al*. 2010).

Studies have shown that the levels of TGF-β in glomerular mesangial cells and the proximal tubules are increased and the level of type IV collagen and FN mRNA is increased in the glomeruli of diabetic patients (Wahab *et al.* 2005). Ziyadeh *et al.* (2000) showed that the level of TGF-β is inhibited by neutralising antibodies and that this prevents the matrix expansion in diabetic patients by reducing the level of type IV collagen and FN mRNA. Several metabolic factors, including elevated glucose, TGF-β, TNF-α have been shown to promote expression of connective tissue growth factor (CTGF) in mesangial cells (Burns *et al.* 2006;
Furlong *et al.* 2007).

Nguyen *et al.* (2008) showed that in animal models CTGF mRNA synthesis is upregulated in various organs, including the kidney and eye. Furthermore, CTGF over-expression in podocytes is critically linked to GBM thickening. Once production of CTGF is induced, it has the ability to orchestrate a host of biochemical and structural changes, namely increased production of FN (Weston *et al.* 2003) and type IV collagen (Zhou *et al.* 2004), induction of plasminogen activator-1 (Wahab *et al.* 2001), increased TIMP-1 and decreased matrix degradation
(Okada *et al.* 2005). All of these processes contribute to GBM thickening in diabetic patients, and hence to DN. In animal models, treatment of diabetic mice with CTGF-antisense oligonucluotides attenuated the progression of DN (Guha *et al.* 2007). Recently,
Nguyen *et al.* (2008a) showed that high plasma CTGF levels were independent risk factors of ESRD and mortality in human type 1 diabetes.

Liu *et al.* (2007) showed that in animal models, accelerated deposition of type IV collagen is present even in the microalbuminuric phase of diabetic kidney disease.
Mason and Wahab (2003) showed an increased deposition of type IV and V collagen, fibronectin and laminin in the mesangial matrix (MM) and GBM in patients with diabetic diffuse glomerular sclerosis. However, a substantial subset of type 2 diabetic patients with microalbuminuria and proteinuria have been shown to have normal glomerular structure with or without tubulointerstitial and or arteriolar abnormalities (Christensen 2004). Schiel *et al.* (2003) showed that, as the level of glycaemia improved to normal levels, the concentration of AGE-products (*e.g.* CML, pentosidine) decreased. However, when complications are already present, improvements to glycaemic control do not show a reversal of pathological processes.

The membrane components type IV collagen and FN, are potential markers of the biochemical and cytological processes underlying the development of DN. With this in mind, the components of GBM, which are used as biomarkers of DN in this study, are considered in greater detail in the following sections.

1.4.1 Type IV Collagen

Type IV collagen accounts for approximately 50% of the BM, including the GBM. It is located primarily in the lamina densa where it forms a large three-dimensional network. Type IV collagen does not form fibres, but instead is secreted and assembles as a pro-collagen molecule containing a highly cross-linked 7S domain. The molecule is known to self-assemble into stabile lattice-like three-dimensional network through interactions at its C- and N-terminal noncollageneous domains. Four molecules of type IV collagen are linked via the N-terminal region (LeBleu *et al.* 2007). Figure 3 shows the structure of collagen protomers and its assembly into the collagen IV suprastructure.

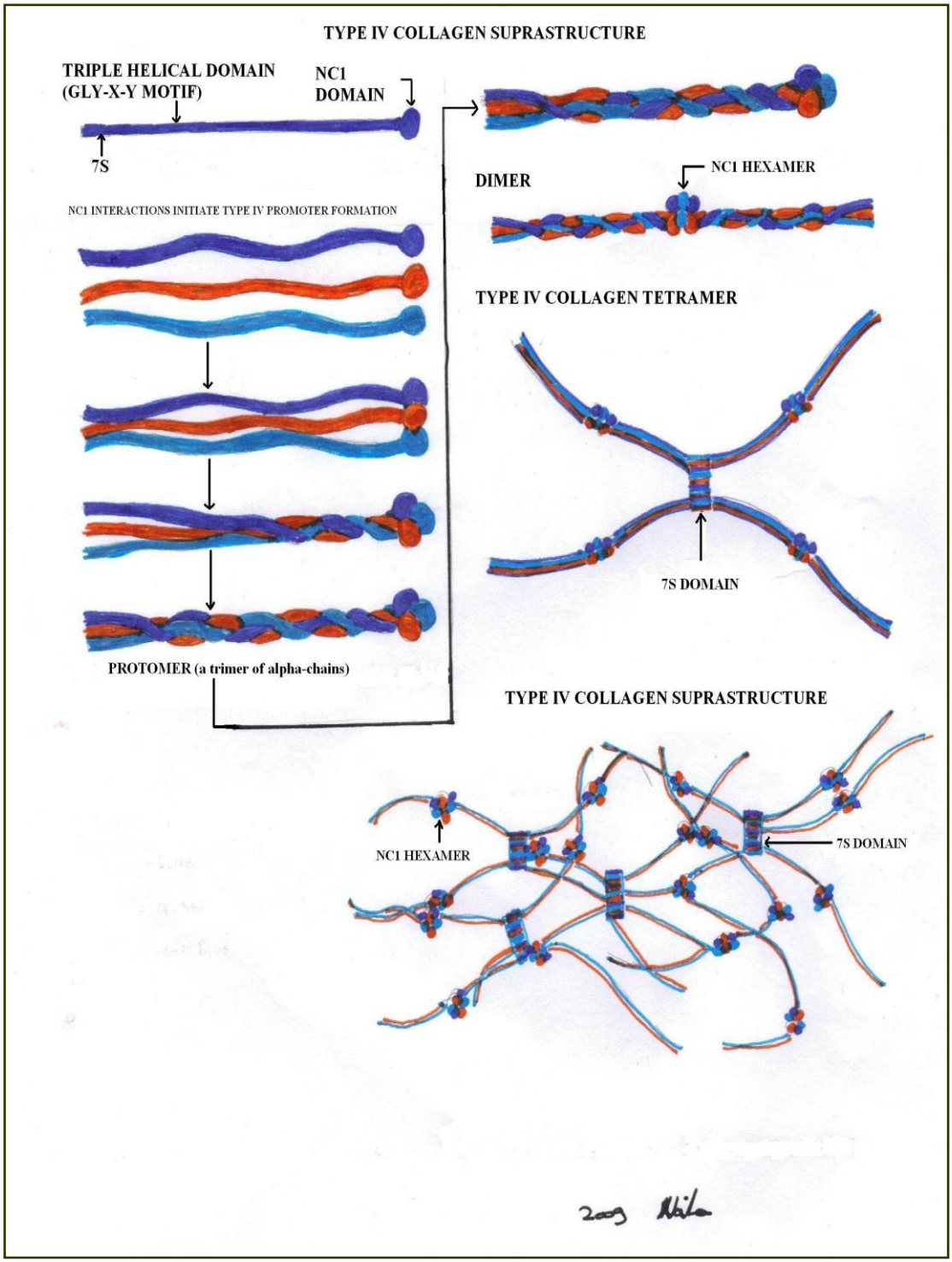

Figure 3. Type IV collagen protomers and assembly into the collagen IV superstructure that forms a supportive lattice in the basement membrane. Adapted from Raghu K. (2003).

Studies have shown that elevated synthesis or increased turnover of type IV collagen might be involved in progression of diabetic microangiopathy, characterised by the thickening of basement membranes. Increased levels of type IV collagen have been shown in diabetic patients compared to healthy controls, and urinary type IV collagen levels increase gradually as DN progresses (Kotajima *et al.* 2000; Watanabe *et al.* 2000; Cohen *et al.* 2001; Tan *et al.* 2002; Tashiro *et al.* 2004; Sanna-Cherchi *et al.* 2007). Not all patients with MA progress to declining renal function. Some patients who develop nephropathy do not develop MA. Therefore, urinary type IV collagen concentration is a potentially useful and non-invasive marker for the development of diabetic microangiopathy and may represent a reliable indicator of the early onset and progression of DN.

1.4.2 Fibronectin (FN)

Fibronectin (FN) is a 440kDa extracellular matrix glycoprotein that binds to integrins (membrane spanning receptor proteins). Fibronectin exists as a dimer, with disulfide bonds linking two nearly identical monomers. It also binds to ECM components (*e.g.* collagen, fibrin and heparan sulfate proteoglycans (HSPG) (Pankov and Yamada 2002). The glycoprotein is produced mainly in the liver (soluable FN) and is also secreted in a soluble form by vascular endothelial cells and platelets (Cho and Mosher 2006) to be assembled into an insoluble matrix (insoluble FN) (Florian *et al.* 2003).

Fibronectin is one of the first structural macromolecules deposited during embryonic development, forming a matrix which allows for the initial organisation to be replaced later by an organ–specific matrix. Its prime role is to act as an adhesive protein attaching cells to a variety of matrices. It is involved in several vascular processes, including structural support, coagulation, platelet function, tissue repair and enhancing the adhesion of erythrocytes to the vascular endothelium. Fibronectin levels increase with age and in age-related processes such as type 2 diabetes, atherosclerosis and osteoarthritis (Mao and Schwarzbauer 2005; Williams *et al.* 2008). Figure 4 shows the structure of FN.

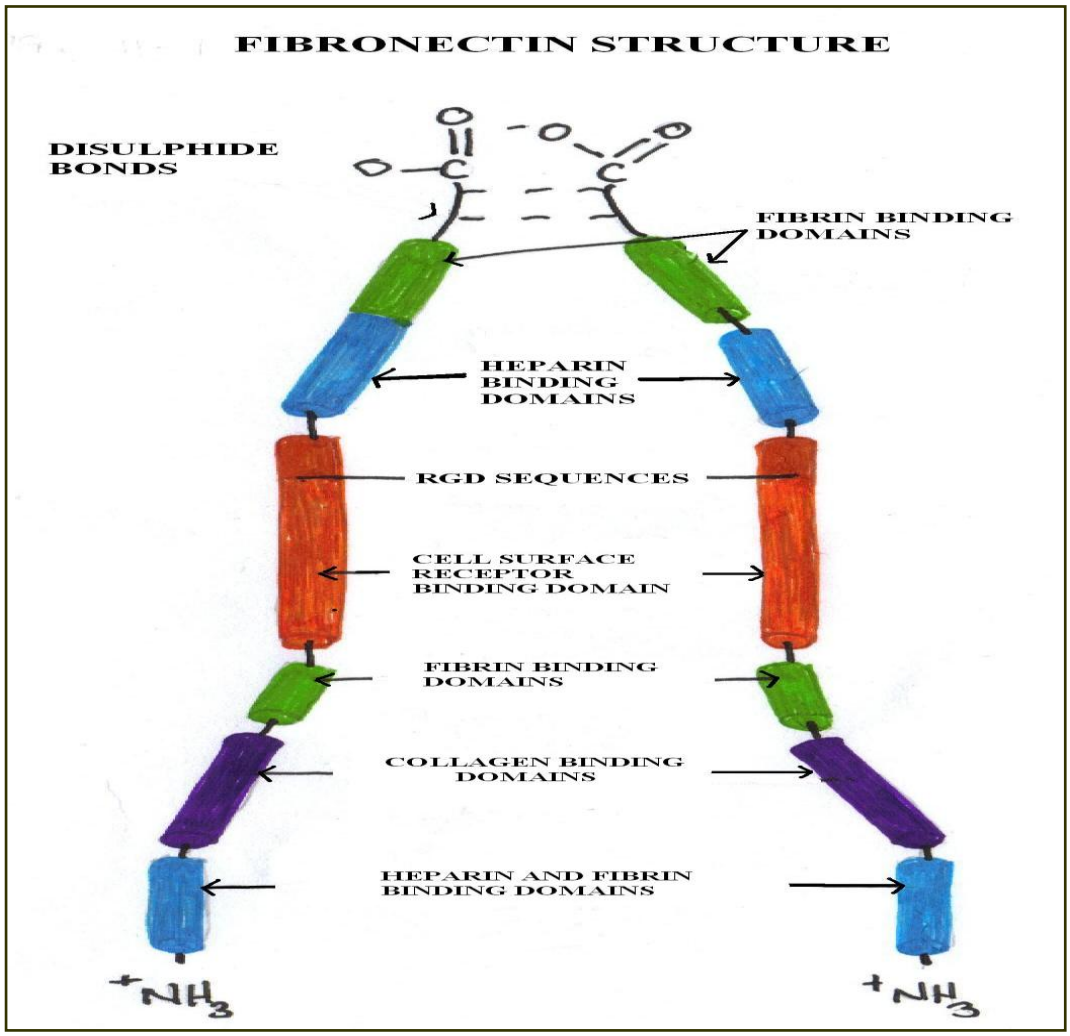

Figure 4. Schematic diagram of fibronectin. Adapted from www.ncbi.nlm.nih.gov.

Elevated plasma levels of FN have been reported in diabetic individuals, as well as in patients with collagen vascular disorders, acute trauma and rheumatoid vasculitis. This may reflect injury to blood vessels (Stanley *et al.* 2008; Stoppacciaro *et al.* 2008). Circulating FN levels may reflect changes to the ECM and vessel wall degradation. Kanters *et al.* (2001) demonstrated that circulating cellular FN may be used as a marker of endothelial cell activation common in diabetes. Wang *et al.* (2002) and Weigert *et al.* (2003) also showed that high glucose increases the production of FN production on mesangial cells. Recently, Cherian *et al.* (2009) showed that the level of FN expression could be decreased with tight glycaemic control in the retinal and renal cells of mice.

While collagen IV and FN are potential new biomarkers of DN, at present DN is managed clinically by the use of more classic markers of glycaemic control and renal function. These markers are described in the following sections.

1.5 CLASSICAL MARKERS OF GLYCAEMIC CONTROL

The results of the DCCT and UKPDS trials have shown that tight glycaemic control significantly reduces the incidence of microvascular complications in both type 1 and type 2 diabetes (DCCT 1993; UKPDS 1998). Furthermore, the lowering of blood pressure also reduces the risk of diabetes-associated complications (Bloomgarden 2001; King *et al.* 2005; ADA 2010). The most widely used markers of glycaemic control of relevance in the clinical setting are discussed below.

1.5.1 Glycohaemoglobin

Haemoglobin (Hb) is the oxygen-transport protein found in red blood cells (RBC). Red blood cells have a circulating half-life of 120 days. The RBC membrane is permeable to glucose and in the presence of circulating glucose, Hb is easily glycated. The glycated form is denoted as haemoglobin A_{1C} (HbA_{1C}). This product is similar to Hb except that it is irreversibly glycated at one or both N-terminal valine residues. Glycation at other portions of the Hb molecule is referred to as non-A_{1C} glycohaemoglobin. All these glycated forms together constitute the total glycohaemoglobin (Goldstein *et al.* 2004).

Regarded as the gold standard for measurement of glycaemic control, HbA_{1c} measurements have proven invaluable in the management of subjects with diabetes. Normal individuals without diabetes have HbA_{1c} value in the range 4% to 5.9%. According to ADA guidelines (ADA 2010), a diagnosis of diabetes is based on HbA_{1c} values greater than or equal to 6.5%. The level of HbA_{1c} is used as a predictor of diabetes complications and studies have demonstrated a reduced risk of complications with lower HbA_{1c} values (DCCT 1993; UKPDS 1998). The goal of glycaemic therapy is to maintain HbA_{1c} levels below 7%. Patients with consistently elevated values above 8% require intensive monitoring as elevated glycohemoglobin levels are strong risk factors for cardiovascular events and other diabetic complications (DCCT 1993; Rohlfing *et al.* 2000; Droumaguet *et al.* 2006).

The use of HbA_{1c} has proven useful for the management of individuals with diabetes. However, clinicians need to be aware that several factors contribute to discrepant results that may affect patient management. In general, all conditions involving diminished RBC survival contribute to lower levels of $HbA_{1c.}$ Patients with iron-deficiency anaemia may show elevated HbA_{1c} values (El-Agouza *et al.* 2002; Kilpatric *et al.* 2008). Abnormal haemoglobin variants in ethnic groups may cause falsely elevated or decreased values (Bry *et al.* 2001; Friess *et al.* 2003; Lee *et al.* 2007).

1.5.2 Glycated Albumin (GA)

Albumin is the most abundant circulating plasma protein, accounting for approximately 60% of the total plasma protein fraction, with a circulating half-life of approximately 13 to 17 days. Under conditions of persistent glycaemia, albumin is readily glycated. The concentration of glycated albumin (GA) is a valuable indicator of recent ambient glycaemia (Cohen *et al.* 2006; Pu *et al.* 2006). Under certain circumstances, primarily in states of recent poor glucose control, levels may be elevated despite normal concentrations of glycohaemoglobin, consistent with the shorter half-life of albumin.

Glycated albumin is better than HbA1c for indicating short-term glycaemic control because of the shorter half-life (Takahashi *et al.* 2007; Yoshiuchi *et al.* 2008; Nagayama *et al.* 2009). Therefore GA is often used to monitor pregnant diabetic women (Roohk & Zaidi 2008; Hashimoto *et al.* 2008). Patients who experience a hyperglycaemic episode show a rise in glycoalbumin earlier than a rise in glycohaemoglobin. Salazar *et al.* (2001) and Cohen *et al.* (2003) showed that glycated albumin induces oxidative stress, promotes phosphorylation of the extracellular signal regulated kinase (ERK) and increases production of TGF-β1, nuclear translocation of transcription factors nuclear factor (NF)-κβ and activator protein (AP)-1. In animal models, glycated albumin was shown to affect the permselectivity of the glomerulus (Londono & Bendayan 2005). Up-regulation of PAI-1 in diabetic animal models was shown to cause extra-cellular matrix accumulation, contributing to DN (Lee *et al.* 2004). In patients with type 2 diabetes increased GA values were linked to increased risk of CAD (Pu *et al.* 2007). Thus there are indications that glycated albumin may be directly linked to some of the complications of diabetes.

1.6 MARKERS OF RENAL FUNCTION

In addition to clinical markers of glycaemic control, diabetic patients are monitored for the onset of diabetic nephropathy using clinical indicators of renal function. Declining renal function in the diabetic milieu is a strong predictor of mortality. Patients with compromised renal function have poor quality of life and increased risk of cardiovascular events. The gold standard of assessing renal function is the measurement of glomerular filtration rate (GFR). This process is tedious and involves expensive machinery and technical expertise. It is for this reason that surrogate markers of renal function are commonly used in the clinical setting to evaluate and monitor patients. Those routinely used for patient management are discussed below.

1.6.1 Serum and urine creatinine

Phosphocreatine serves as an energy source in tissues and organs that have various degrees of activity, *e.g.* skeletal muscle and brain. Creatinine is the breakdown product of phosphocreatine and is formed at a stable rate depending on the level of physical activity and muscle mass. The creatinine generated is filtered out of circulation by the kidney. Since there is minimal tubular re-adsorption of creatinine, a compromise of kidney function results in the appearance of high levels of serum creatinine.

Creatinine is an amino acid derivative with a molecular mass of 113 Da that is secreted in the kidney by the proximal tubular cells and easily filtered by the glomerulus under normal conditions. Tubular secretion varies amongst individuals especially in those with a moderate reduction of glomerular filtration rate (GFR). Various factors, such as age, gender, ethnicity, protein intake, and amount of lean muscle mass affect creatinine concentration. Furthermore, the measured value may remain within acceptable patient reference ranges despite compromised renal function in patients with low muscle mass, as in the elderly and young children, or be falsely elevated in patients with higher muscle mass, especially adult males of African origin in the presence of normal renal function (Rule *et al.* 2004; Hsu *et al.* 2008).

Creatinine clearance is an inaccurate measure of renal function since variable tubular secretion impacts on its accurate measurement. Urine creatinine is inaccurate due to collection errors and poor patient compliance (Coresh *et al.* 2002). Certain drugs (*e.g*. cimetidine) inhibit secretion, reduce clearance and raise serum values without affecting the GFR. Dietary intake of protein and muscle mass (especially males) affect the value of creatinine. Furthermore, the level of creatinine varies due to geography, ethnicity, age and racial groups (Cirillo *et al.* 2005; Poggio *et al.* 2005; Ma *et al.* 2006). Extrarenal elimination of creatinine in patients with low GFR is increased due to bacterial degradation (Stevens & Levey 2005) and the lack of a single standardised reference range makes the use of a single creatinine value to estimate a normal GFR from an abnormal one misleading in the clinical setting (Levey *et al.* 2000; Myers *et al.* 2006). These limitations need to be borne in mind in the clinical interpretation of creatinine concentrations.

1.6.2 Albumin excretion rate (AER)

Diabetic nephropathy has been characterised by different stages of albumin excretion ranging from normoalbuminuria, MA (AER 20 ≤ 200 μg/min) to macroalbuminuria or proteinuria (AER > 200 μg/min), the latter corresponding to severe histopathology (Hillege *et al.* 2002; Steinke *et al.* 2005).

For type 2 diabetic patients, development of nephropathy typically occurs within five to ten years of diagnosis in about 25% of patients (Adler *et al.* 2003) and cardiovascular risk increases 2 to 4 fold (Calvo *et al.* 2006). A progression rate of greater than 80% from MA to gross proteinuria in type 1 individuals has lead to the broad acceptance of MA as a predictor of increased DN risk (Caramori *et al.* 2000; K/DOQI 2007).

1.6.3 Glomerular filtration rate (GFR)

The glomerular filtration rate (GFR) is defined as the amount of fluid filtered through the glomerulus into the Bowman's capsule within a unit of time. Normally, GFR is measured by the use of a labelled marker and its complete clearance per unit time. The following equation (Equation 1) is used to calculate GFR.

$$GFR = U \times V \div P \qquad \textbf{Equation 1}$$

U = Concentration of substance in urine
V = Flow rate of urine
P = Average plasma concentration

Characteristics of an ideal marker are that it is derived endogenously, undergoes filtration and elimination only by the kidney and be neither excreted nor reabsorbed by the renal tubules. The gold standard for the measurement of GFR is the measurement of renal clearance of inulin, a plant derived polymer consisting of several fructose units containing a terminal glucose (Sirwal *et al.* 2004; Roberfroid 2007). Isotopic methods have produced results of comparable accuracy, namely renal or plasma clearance of [^{51}Cr]-EDTA, [^{131}I]-diatrizoate, [^{131}I]-iothalamate, [^{125}I]-iothalamate or [^{99m}Tc]-DTPA and iohexol (Peters *et al.* 2009). The plasma clearance method is inaccurate especially in individuals where the GFR is low or if oedema is present. The main disadvantage of these methods is that they are time-consuming and cumbersome to perform and require highly skilled staff and expensive equipment (Fontsefe *et al*. 2006). Patients with MA can show a significant decline in GFR (Amin *et al.* 2005). Perkins *et al.* (2007) showed that early renal function decline occurred in 31% of microalbuminuric patients despite normal or elevated renal function at baseline.

1.6.4 Estimated glomerular filtration rate (eGFR)

In accordance with guidelines of the National Kidney Disease Education Program (NKDEP 2008), National Institutes of Health (NIH) and the Kidney Disease Outcomes Quality Initiative (K/DOQI 2002), many laboratories are reporting an estimated glomerular filtration rate (eGFR) together with serum creatinine values. The eGFR proves useful in the management of patients that have established chronic kidney disease (CKD) as well as in patients that are at risk, namely patients with diabetes, hypertensive patients and those with a familial history of kidney failure (Levey *et al.* 2003; Tidman *et al.* 2008; Premaratne *et al.* 2008).

The most commonly used equation for eGFR is the Modification of Diet in Renal Dialysis, known as the MDRD equation (Equation 2) (Levey *et al.* 1999; Levey *et al.* 2000). This formula incorporates the patient's serum creatinine, sex, age and ethnicity and is reported as mL/min/1.73 m^2 .The 4-variable (4-v) MDRD equation was developed in 1999 based on a larger population group (1628 patients with CKD) than the Cockcroft-Gault (CG) equation discussed below. The MDRD formula adjusts for body surface area and takes into consideration female sex and ethnic background (Black). The formula was revised in 2005 for use with a standardised creatinine assay. The values using the standardised creatinine assay were 5% lower that those reported in the original study, using a non-standardised alkaline picric acid method for serum creatinine (Sjostrom *et al.* 2005; Myers *et al.* 2006).

4-variable MDRD equation:

$$\text{eGFR} = 186.3 \times \text{SCr}^{-1.154} \times \text{AGE}^{-0.203} \times 0.742 \text{ (if female)} \times 1.212 \text{ (if black)}$$

SCr = serum creatinine in mg/dL (multiply by 88.4 to convert to μmol/L)

Equation 2

The Cockcroft-Gault equation (GC equation) (Equation 3) is based on data from 249 non-diabetic hospitalised men with serum creatinine values ranging from 30-130 mL/min. It was developed to estimate creatinine clearance. The formula incorporated age, weight and serum creatinine and has been shown to systematically overestimate GFR due to tubular secretion (Cockcroft and Gault 1976).

Cockcroft-Gault Equation:

$$eGFR_{CC} = [(140 - AGE) \times \text{weight (Kg)}] \div SCR \times 72 \times [0.85 \text{ if female}]$$

(adjusted for body surface area of 1.73 m^2)

SCr = serum creatinine in mg/dL (multiply by 88.4 to convert to μmol/L)

Equation 3

Numerous studies (Lewis *et al.* 2001; Rule *et al.* 2004; Hallan *et al.* 2004; Poggio *et al.* 2005) have validated the use of the MDRD equation in patients both with and without CKD (eGFR values < 90mL/min/1.73 m^2) (Bostom *et al.* 2002; Lin *et al.* 2003) in various population groups including Blacks (Emmanuel *et al.* 2009), Asians (Zuo *et al.* 2005), and renal transplant patients (Poge *et al.* 2006; White *et al.* 2009).

In comparison to the CG equation, the MDRD equation has been shown to be more accurate in evaluating eGFR in older and obese patients (Verhave *et al.* 2005). Both equations have limited accuracy when monitoring young type 1 diabetic patients with CKD in the absence of microalbuminuria as well as potential kidney donors (Ibrahim *et al.* 2005; Gopalakrishnan and Gourabathini 2007). Despite recent reports of mean differences between a measured GFR and MDRD eGFR ranging from –29 to 3 mL/min/1.73m^2 and the potential of misdiagnosing a patient with a mild reduction of GFR as having CKD, the formula has proven useful in identifying patients at risk of developing DN when eGFR of less than 60 mL/min/1.73m^2 were reported (Sarnak *et al.* 2003; Weiner *et al.* 2004).

The performance of the 4-v MDRD and CG equations was evaluated to assess the appropriateness of the formula in Black South Africans (van Deventer *et al.* 2008). Patients with established chronic kidney disease (CKD) or risk factors for developing CKD, such as hypertension, diabetes and HIV were recruited in the study. The revised 4-v MDRD equation without ethnicity factor of 1.212 (Equation 4) and the CG equation after correcting for bias were more appropriate in Black South Africans to estimate GFR than the 4-v MDRD equation.

Revised 4-variable MDRD equation without ethnicity factor:

$$eGFR = 186.3 \times SCR^{-1.154} \times AGE^{-0.203} \times 0.742 \text{ (if female)}$$

SCr = serum creatinine in mg/dL (multiply by 88.4 to convert to μmol/L)

Equation 4

1.7 USE OF RENAL FUNCTION MEASURES IN MONITORING CHRONIC KIDNEY DISEASE (CKD)

Chronic kidney disease (CKD) is defined either by a GFR < 60 mL/min/m^2 or the presence of kidney damage independent of the cause for three or more months (National Kidney Foundation Kidney Disease Outcomes Quality Initiative, K/DOQI 2002; Dirks *et al.* 2005). Moderate to severe CKD is estimated to be found in 15-23% of patients with diabetes (Coresh *et al.* 2003; Middleton *et al.* 2006). Patients that are at increased risk include those 60 years and older, and patients who have hypertension, diabetes, cardiovascular disease and a familial history of kidney disease. Table 4 below shows the GFR values and the stages of CKD, which are used for patient management.

Table 4. Stages of Chronic Kidney Disease (CKD). Adapted from the Kidney Disease Outcome Quality Initiative of the National Kidney Foundation (2002).

Stage	Description	Glomerular filtration rate (GFR) (mL/min/1.73m^2 body surface area)
1	Kidney damage with normal or increased GFR	≥ 90
2	Kidney damage with mildly decreased GFR	60 to 89
3	Moderately decreased GFR	30 to 59
4	Severely decreased GFR	15 to 29
5	Kidney failure (end stage renal disease)	< 15 or dialysis

Current recommendations for management of patients at increased risk include routine monitoring of AER and eGFR based on the MDRD equations (K/DOQI 2002; Chobanian *et al.* 2003; Levey *et al.* 2005; Levey *et al.* 2007). Clinicians face the task of accurately interpreting eGFRs at or near 60 mL/min/1.73 m^2. Patient evaluation should be assessed using overall information. Any of the risk factors such as proteinuria, renal biopsy, and/or abnormal imaging analysis must be interpreted as disease being present, even if eGFRs are reported as ≥ 60 mL/min/1.73 m^2. For patients without risk factors and eGFRs just below 60 mL/min/1.73 m^2, the measured GFR should be used together with risk factors of the disease (Stevens & Levey 2009).

1.8 RATIONALE OF PRESENT STUDY

It is evident from review of the literature that DN is a major microvascular complication arising from diabetes. Numerous studies have shown that the early identification of patients with CKD and the implementation of appropriate treatment is associated with better prognosis and the delay of ESRD (Levey *et al.* 2007). Although microalbuminuria is a marker of compromised kidney function, it lacks specificity for DN. It is for this reason that markers of glycation were investigated, notably urinary type IV collagen, fibronectin and glycated albumin. Several studies have indicated that one or more of these shows promise as an early indicator of DN.

A prospective study was undertaken to determine the feasibility of using glycated albumin, plasma fibronectin and urinary type IV collagen as improved markers of incipient DN in South African Black and Indian patients with type 1 diabetes, as compared to the current standards of clinical care, including GFR and microalbuminuria.

1.9 STUDY HYPOTHESIS

It was hypothesised that glycated serum albumin, plasma fibronectin and urinary type IV collagen, either alone or in combination are better predictors of impaired renal function than microalbuminuria. It was further hypothesised that this relationship differs between South African Black and Indian patient populations.

1.10 AIMS AND OBJECTIVES OF PRESENT STUDY

Aim:

To investigate the feasibility of using serum glycated albumin, plasma fibronectin and urinary type IV collagen as improved markers, relative to MA, of incipient diabetic nephropathy in patients with type 1 diabetes.

Objectives:

1. Identification of eligible study and control individuals and recruitment into study.
2. Development of enzyme-linked immunosorbent assays (ELISA) for the detection of type IV collagen, fibronectin and glycated albumin in blood and urine specimens.
3. Monitoring of patients over a two-year period and controls over a six-month period, measuring urinary type IV collagen, serum glycated albumin and plasma FN.
4. Statistical testing of the correlation of MA, plasma FN, urinary type IV collagen and serum glycated albumin with isotope and estimated GFR in Black and Indian populations.
5. Statistical testing of the ability of plasma fibronectin, serum glycated albumin and urinary type IV collagen to predict isotope and estimated GFR in Black and Indian populations.
6. Statistical testing of the relationships among different measures of renal function.

CHAPTER 2

STUDY DESIGN AND METHODS

2.1 DESIGN OF CLINICAL STUDY

The study was undertaken at the Inkosi Albert Luthuli Central Hospital (IALCH), KwaZulu- Natal. It was a prospective study with sampling of blood and urine at baseline and at follow-up at 6 months, 1 year and 2 years. South African Black and Indian patients attending the IALCH Diabetes Clinic were recruited as study participants. Each of the participants signed informed consent for participating in this study (Appendix 1). The Nelson R. Mandela School of Medicine Ethics Committee approved this study (Appendix 2).

2.1.1 Study subjects

Patients with type 1 diabetes comprised the study group. Patients with type 2 diabetes were excluded because they are more difficult to study in view of the age- and disease-related macrovascular disease and hypertension that are common clinical findings of type 2 diabetes. To date, studies of the effects of a hyperglycaemia on vascular disease, especially the glomerulus in patients with type 1 diabetes are under-represented. At the start of the study, there was a total of 83 patients (Black 54; 24 male, 30 female; Indian 29; 14 male, 15 female). At 2-year follow-up, there was a total of 58 patients (Black 34; 17 male, 18 female; Indian 23; 11 male, 12 female).

2.1.2 Control subjects

Normal healthy Black and Indian subjects comprised the control group. Control subjects were not on any chronic medication and had no diabetic symptoms. All subjects gave their signed informed consent for participation in this trial. Subjects were chosen as they were healthy, age matched to study participants and not on any chronic medication (Appendix 1). At the start of the study there was a total of 73 patients (Black 45; 16 male, 29 female; Indian 28; 12 male, 16 female). At 6-month follow-up there was a total of 36 patients (Black 16; 4 male, 12 female; Indian 20; 10 male, 10 female).

2.1.3 Criteria for inclusion and exclusion

The inclusion criteria for patients were:

1. Type 1 diabetes, diagnosed clinically as absolute insulin dependency within 1 year of diagnosis and/or presentation at less than 25 years of age with ketoacidosis.

   The diagnosis of type 1 diabetes was based on clinical demonstration of complete beta cell failure. This was defined as absolute insulin deficiency within 1 year of diagnosis. These subjects would develop ketoacidosis in the absence of insulin therapy. In addition, if the person was in the age range of onset of type 1 diabetes and insulin deficiency was shown by the fact that ketoacidosis was present at the time of diagnosis, then the classification was type 1. Beta cell specific autoantibodies were done on most of the subjects, but not all and the clinical markers correlate with the presence of beta-cell autoantibodies.

2. The presence of established microvascular disease or hypertension was not considered an exclusion criterion.

The exclusion criteria for patients were:

1. Type 2 diabetes or other forms of diabetes.
2. Significant co-morbid conditions.
3. Infection with HIV.
4. Hypoalbuminaemia (< 40 g/L).
5. Non-diabetic renal disease.
6. Active urinary tract infection.

2.1.4 Discontinuation of patients

Patients who developed any conditions that, in the opinion of the investigators, made it unsafe for subjects to continue in the study and those who developed medical conditions that formed part of the exclusion criteria were withdrawn from the trial. Other conditions for premature withdrawal from the study included patients that were involved in accidents, passed away or sero-converted to HIV. Subjects that missed two consecutive follow-up visits were also excluded.

2.1.5 Routine analyses performed for each visit

At each visit, the results of routine parameters were recorded. These were:

- Height.
- Weight.
- Body Mass Index (BMI).
- Pulse.
- Waist and hip measurements.
- Waist-to-hip ratio (WHR).
- Insulin regimen, total daily dose.
- Additional therapy.
- Blood pressure (BP).
- Fasting plasma glucose (FPG).
- Serum albumin.
- Urine microalbumin.
- Glycated haemoglobin (HbA_{1c}).
- serum creatinine.

Weight (Kilograms) was measured on a scale (NAGATA Scale Co, Taiwan) and height measured on a clinical stadiometer (centimetres). Waist measured mid-way between the lower costal margin and the iliac crest with a non-stretch tape. Hip circumference measured around the greater trochanters of the femur, with a non-stretch tape. Blood pressure and pulse were measured using a MEDIANA (Korea) analyser after a 5-minute rest period. In addition, glomerular filtration rate (GFR) was measured yearly by technetium-99^{m}-diethylene triamine pentacetic acid [^{99m}Tc]-DTPA renography as described by Ma *et al*. (2007).

Isotope GFR was performed at the Department of Nuclear Medicine at IALCH by a qualified radiographer. Fasting plasma glucose (FPG) was assayed by a hexokinase method. Serum creatinine was assayed by a kinetic Jaffé method standardised against isotope dilution-mass spectrometry (ID-MS). Albumin was assayed by a colorimetric method. Microalbumin was assayed by an immunoturbidometric method. All assays were performed on a Hitachi 917 analyser (Roche Diagnostics, Indianapolis, USA). Haemoglobin A_{1c} (HbA_{1c}) was assayed using ion-exchange high-performance liquid chromatography on a VARIANT II analyser (BIO-RAD, CA). Urinary protein was assayed using SIEMENS Multistix ® 10SG strips (Siemens Healthcare Diagnostics Inc, Tarrytown, USA). Clinical proteinuria was indicated if protein excretion was greater than 0.5 g (500 mg) per day (test strip result of 0.3 g/L). Patients were screened for proteinuria by dipstick; if this was negative, then MA was tested. In control subjects it was assumed that proteinuria was absent so all controls were tested for MA.

2.1.6 Sample collection and storage

At each visit, blood and urine specimens were collected from each patient as follows: two whole blood samples were collected in ethylenediaminetetraaceticacid (EDTA). In addition, two serum samples, one sodium fluoride (glucose) sample and a spot urine sample were collected. Bloods were collected aseptically using standard phlebotomy techniques and collected in the order: serum, EDTA, glucose. The EDTA and sodium fluoride tubes were gently mixed to prevent clot formation while the serum specimens were allowed to stand at room temperature (20 to 25°) for 15 minutes to clot completely. Spot urine samples were collected aseptically and frozen within two hours of collection at –20 °C. Serum, EDTA and glucose specimens were centrifuged (Biofuge primo, Heraues) at 1000 g for five minutes. Plasma from the EDTA and sodium fluoride tubes were separated into Nunc cryovials (Denmark), appropriately labelled and frozen at – 20°C.

2.3 COLLAGEN IV H ASSAY KIT

The Collagen IV H assay kit (Exocell, Philadelphia, USA) was used to determine levels of urinary type IV collagen in patient and control samples. The kit contained reagents ready for use and the assay was performed according to the manufacturer's instructions. The EIA wash buffer (0.15 M NaCl, 0.01M triethanolamine at pH 6.8, 0.05% Tween 20, 0.05% Proclin 300 (Supelco, Bellefonte, Philadelphia, USA) was constituted according to the manufacturer's instructions and made to a 10 × concentrate. This was used for all assay plate washing.

2.3.1 Assay principle

The Collagen IV H assay is a competitive indirect ELISA. Briefly, equal volumes of samples containing collagen type IV and rabbit anti-human collagen type IV were added to vials (Figure 5). Following incubation overnight (18 to 21 hours) at room temperature (20 to 25°C), the pre-incubation step allowed binding of the antibody and antigen in solution, thereby limiting binding to coated antigen when competing antigen was present in low concentrations. Following overnight incubation an aliquote of the solution was added to the well of the ELISA plate. The antibody binds either to the collagen IV in solution or to that coating the plate (Figure 6). This allowed measurements of collagen type IV in unconcentrated urine samples (Cohen *et al.* 2001). The addition of a chromogenic substrate, 3,3',5,5' tetramethyl-benzadine (TMB, Exocell) allowed for colour development. The reaction was stopped by the addition of 2 N sulphuric acid and the absorbance in the wells was read at 450 nm. Colour intensity of the wells was inversely proportional to the logarithm of human collagen type IV concentration in the sample. The assay was sensitive to 0.0024 µg/mL. Intra-assay and inter-assay coefficients of variation were < 10 %.

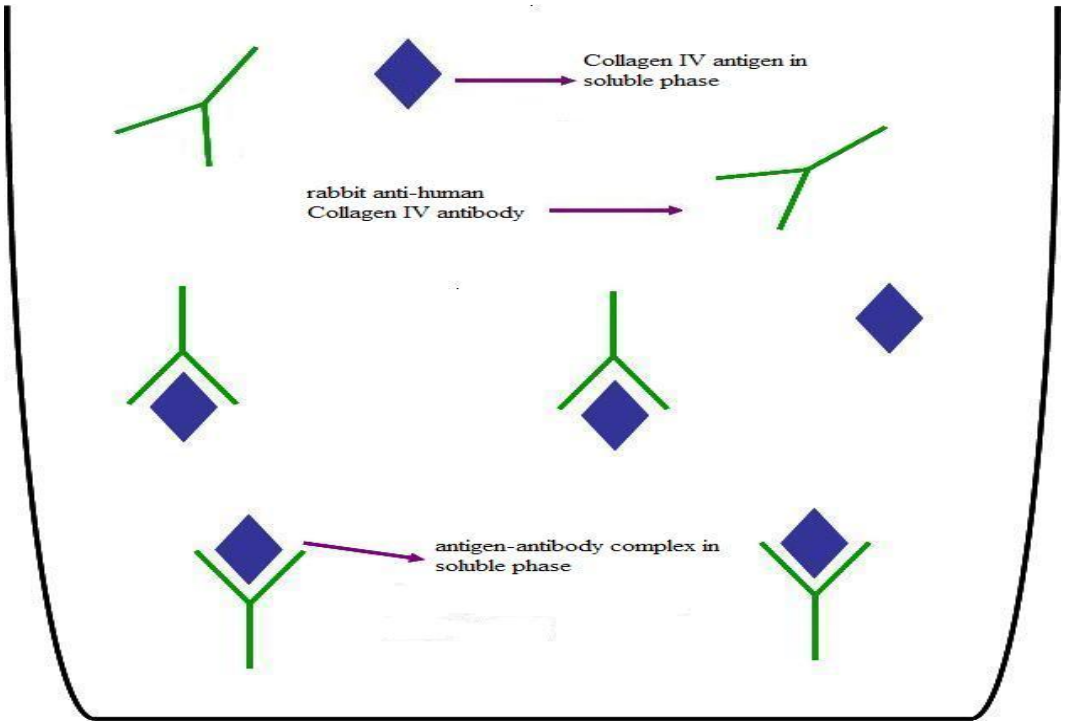

Figure 5:Schematic diagram showing the antigen-antibody complex formation following overnight incubation. Adapted from www.genwaybio.com.

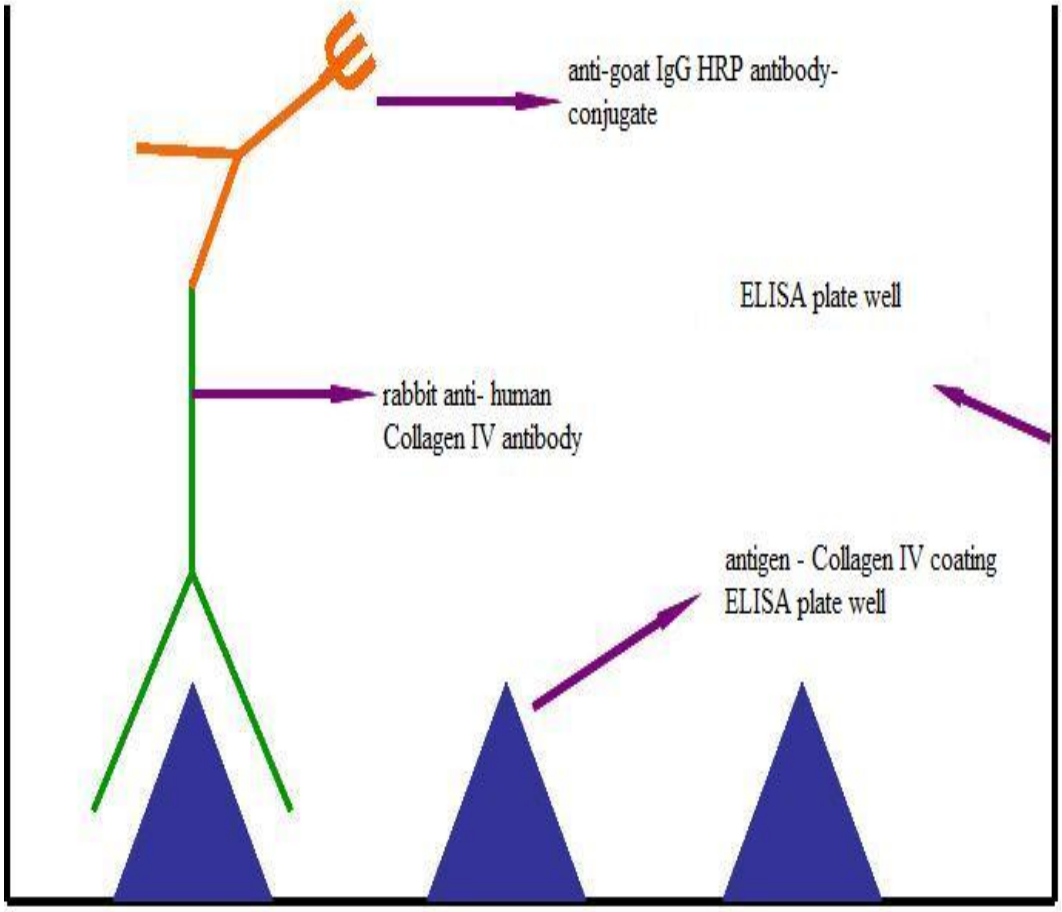

Figure 6: Schematic diagram showing the binding of uncomplexed collagen IV antibody to collagen IV antigen coated on ELISA microtiter plates. Adapted from www.genwaybio.com**.**

2.3.2 Assay of standards and samples

All assays were performed in 96-well microtitre plates pre-coated with collagen type IV antibody (Exocell, Philadelphia, USA) at room temperature (20 to 25°C). Plates were incubated in a moistened plastic chamber with tightly fitting lid unless otherwise specified. The stock human collagen type IV standard (Exocell, 20 µg/mL) was diluted 1 in 8 using EIA diluent (360 µL: 2 520 µL). The dilution yielded a final collagen IV concentration of 2.5 µg/mL.

The standard was serially diluted one in two using EIA diluent to yield the following final collagen IV concentrations as follows: 1.25, 0.625, 0.313, 0.156, 0.078 and 0.039 µg/mL.
To each vial was added a volume of 625 µL of goat anti-human collagen IV antibody and the tubes were vortexed briefly. The final volume (1 250 µL) for each standard concentration was sufficient for six wells (200 µL/well). The standards were incubated overnight (21 hours) at room temperature and assayed the following day.

Samples (patient and control) were removed from the freezer and thawed for 10 minutes in a water bath containing tap water at room temperature (20 to 25°C). Samples were centrifuged (Biofuge primo, Heraues) for five minutes at 1000 g. Equal volumes of sample supernatant and primary antibody (Exocell) were incubated in an Eppendorf tube (Denmark) and allowed to react overnight (18 to 21 hours).

To start the assay, pre-coated plates were washed 10 times using EIA buffer (as specified previously) and blotted by inverting onto absorbent paper towel. A total volume of 200 µL of the standard or sample mixtures were added to the wells. Each standard was pipetted into six wells, samples were tested in duplicate. The plates were incubated for one hour. Following incubation, the plates were washed as described above, 100 µL of rabbit anti-goat IgG horseradish peroxidase (HRP) conjugate was added to each well and the plates incubated for one hour.

The plates were washed as described above and 100 µL of TMB substrate was added to each well. The plates were incubated for a further five to ten minutes for colour development. The colour development was stopped by the addition of 100 µL of acid stopper (2 N sulphuric acid). The assay plate was read on a PowerWaveXS microplate reader (BIOTEK, Lionheart Technologies Inc, USA) at 450 nm with blanking against wells A1 and A2. The blank wells containing EIA diluent were used as a negative control to ensure that there was no non-specific binding of reagents to the coated plate.

A standard curve was plotted on a log scale, using the KC4 software provided with the microplate reader to generate a dose-response curve. The average optical density (OD) of six wells for the standards were used to generate a standard curve, which was used for subsequent assays to determine the concentration of collagen type IV in each sample. In subsequent assays, one of the dilutions of the stock standard used to generate the standard curve was used as internal quality control to check assay validity. Blanks were included in each assay to ensure that there were no non-specific reactions occurring between reagents. Patient samples tested previously were run with the next assay to confirm assay reproducibility.

2.4 HUMAN FIBRONECTIN ASSAY

The human fibronectin (h fibronectin) assay (Exocell, Philadelphia, USA) was used according to manufacturer's instructions. The assay was performed in triplicate for both standards and samples (patients and controls).

2.4.1 Assay principle

The Exocell h fibronectin assay is a sandwich ELISA with a sensitivity range of approximately 0.2 to 5 mg/mL. The assay is dependent on the binding of the human fibronectin in the standard or sample to pre-coated microtitre wells (Figure 7). Briefly, samples were prediluted using EIA buffer. Following an initial incubation, a washing step removed unbound reactants. Anti-human fibronectin-HRP conjugate was added to each well and incubated. Conjugate binds to the human fibronectin that was initially bound to the solid phase. A washing step removed unreacted conjugate. The addition of a chromogenic substrate, (TMB) allowed for colour development. The reaction was stopped and the absorbance in the wells was read at 450 nm. Colour intensity in the wells was directly proportional to the logarithm of human fibronectin concentration in the sample. The assay was sensitive to 0.125 mg/mL. Intra-assay and inter-assay coefficients of variation were < 10 %.

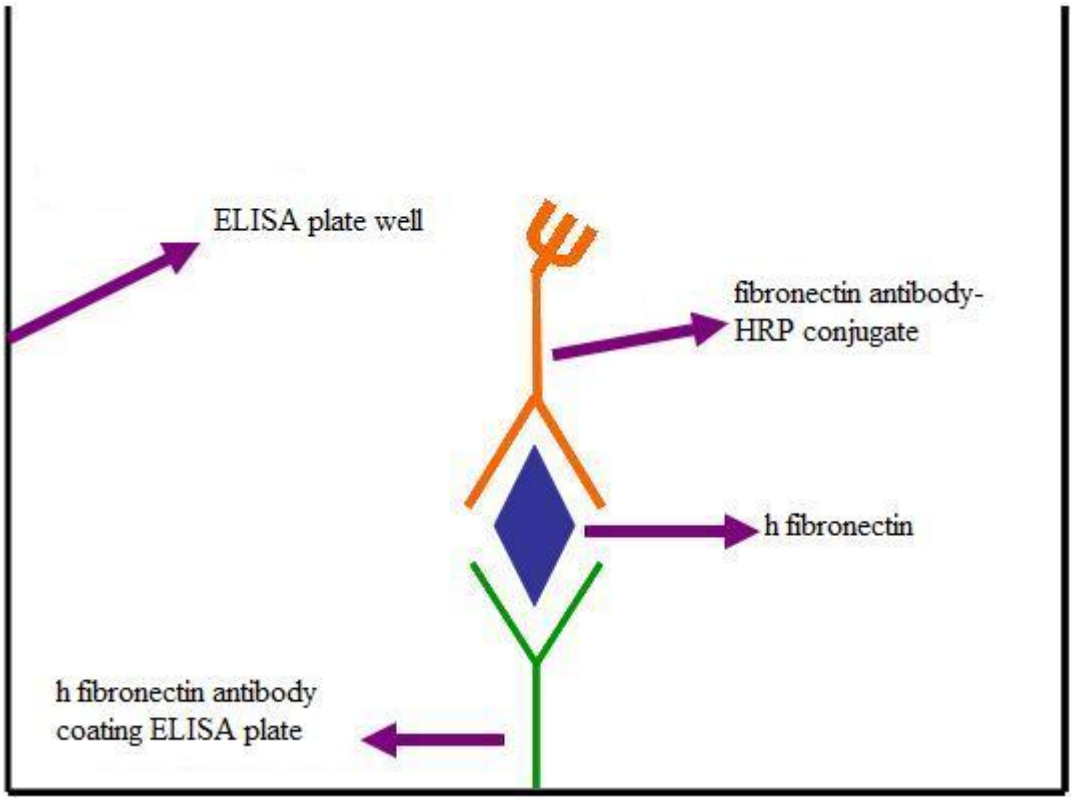

Figure 7: Schematic diagram showing the binding of human fibronectin to precoated microtiter plates. Adapted from www.genwaybio.com.

2.4.2 Assay of standards and samples

All assays were performed in 96-well microtitre plates, pre-coated with h fibronectin antibody (Exocell, Philadelphia, USA) at room temperature (20 to 25°C). Plates were incubated in a moistened plastic chamber with tight fitting lid unless otherwise specified. The stock h fibronectin standard (Exocell, 4 mg/mL) was serially diluted one in two using h fibronectin reaction buffer (Exocell) to yield the following final h fibronectin concentrations: 4 (undiluted), 2, 0.1, 0.5, 0.25 and 0.125 mg/mL. Samples were removed from the freezer and thawed for ten minutes in a water bath containing tap water at room temperature (20 to 25°C) and centrifuged (Biofuge primo, Heraues) for five minutes at 1000 g. Samples were pre-diluted (5 µL/mL) with h fibronectin reaction buffer.

To start the assay, pre-coated plates were washed 10 times using EIA buffer (as specified previously) and blotted by inverting onto absorbent paper towel. A total volume of 100 µL of standard or sample was added to the wells after vortexing the tube. Each standard was pipetted into six wells. Samples were tested in triplicate. The plates were incubated for 30 minutes. Following incubation, the plates were washed as described above and 100 µL of anti-human fibronectin horseradish peroxidase (HRP) conjugate was added to each well. The plates were then incubated for 30 minutes. The plates were washed as described previously and 100 µL of TMB substrate was added to each well. The plates were incubated for a further five to ten minutes for colour development.

The reaction was stopped by the addition of 100 µL of 2 N sulphuric acid. The assay plate was read on a PowerWave XS microplate reader (BIOTEK, Lionheart Technologies Inc, USA) at 450 nm blanking against wells A1 and A 2. The blank wells contained human fibronectin reaction buffer and were used as a negative control to ensure that there was no non-specific binding of reagents to the coated plate.

A standard curve was plotted on a log scale, using the KC4 software provided with the microplate reader. The average optical density (OD) of six wells for the standards was used to generate a standard curve, which was used for subsequent assays to determine the concentration of human fibronectin in samples. In subsequent assays, one of dilutions of the stock standard used to generate the standard curve was used as internal quality control to check assay validity. Blanks were included in each assay to ensure that there were no non-specific reactions occurring between reagents. Patient samples tested previously were run with the next assay to confirm assay reproducibility.

2.5 GLYCABEN ASSAY FOR GLYCATED ALBUMIN

The GLYCABEN assay (Exocell, Philadelphia, USA) was used to assay glycated albumin in serum. The kit contained reagents ready for use and the assay was performed according to the manufacturer's instructions. The EIA wash buffer (as specified previously) was constituted according to the manufacturer's instructions and used to perform the plate washings.

2.5.1 Assay principle

The GLYCABEN assay is a direct ELISA in which glycated albumin in human plasma binds to an immobilised monoclonal antibody that specifically recognises the glycated moieties on human albumin (Figure 8). Plates were washed with EIA buffer (as specified previously) followed by the addition of anti-human serum albumin-horseradish peroxidase (HSA-HRP) conjugated polyclonal antibody. The enzyme conjugate added bound to the glycated albumin already bound to the solid phase. After washing and TMB, colour allowed to develop. The reaction was stopped with the addition of 2 N sulphuric acid and the absorbance was read at 450 nm. The assay was sensitive to 0.125 mg/mL. Intra-assay and inter-assay coefficients of variation were < 10 %.

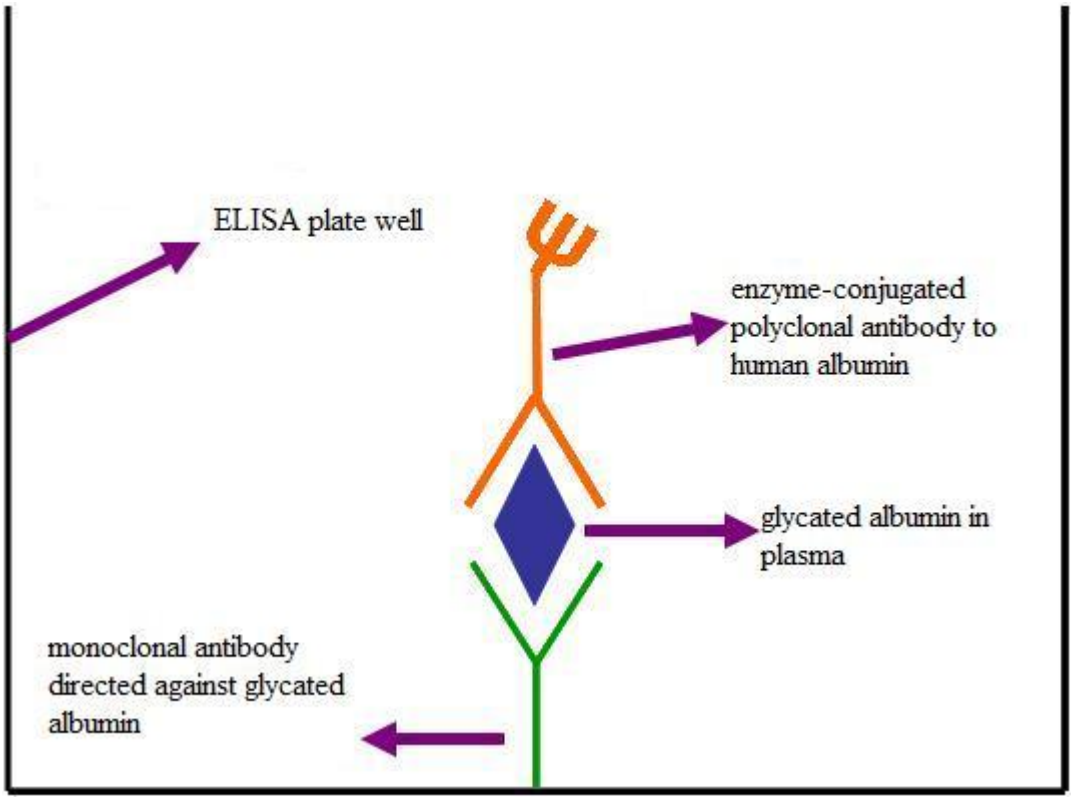

Figure 8: Schematic diagram showing the binding of glycated albumin to immobilised monoclonal antibodies on the ELISA plate. A secondary enzyme-conjugated polyclonal antibody directed against human albumin allows for detection of glycated albumin. Adapted from www.genwaybio.com.

2.5.2 Assay of standards and samples

All assays were performed in 96-well microtiter plates pre-coated with monoclonal antibody to glycated albumin (Exocell, Philadelphia, USA) at room temperature (20 to 25°C) and incubated in a moistened plastic chamber with tight fitting lid unless otherwise specified. The stock glycated albumin standard (Exocell, 1.5 mg/mL) was serially diluted one in two using GLYCABEN diluent (Exocell) to yield the following final glycated albumin concentrations as follows: 1.5 (undiluted), 0.75, 0.375, 0.1875, 0.09375 mg/mL. Samples to be tested were removed from the freezer and thawed for ten minutes in a water bath containing tap water at room temperature (20 to 25°C) and centrifuged (Biofuge primo, Heraues) for five minutes at 1000 g Sample supernatants were pre-diluted (5 µL/mL) with GLYCABEN reaction buffer. Quality controls provided in the kit were used in each assay.

To start the assay, pre-coated plates were washed 10 times using EIA buffer (as specified previously) and blotted by inverting onto absorbent paper towel. A total volume of 50 µL of GLYCABEN reaction buffer was added to all wells. This was followed by the addition of 100 µL of standard or sample to the wells. Each standard was pipetted into six wells; samples were tested in duplicate. The plates were incubated for 30 minutes. Following incubation, the plates were washed as described above, 100 µL of anti-human serum albumin-horseradish peroxidase (anti-HSA-HRP) conjugate were added to each well and the plates incubated for a further 30 minutes. Plates were washed and 100 µL of TMB substrate was added to each well. Plates were incubated for a further five to ten minutes for colour development. The reaction was stopped by the addition of 100 µL of 2 N sulphuric acid.

Assay plates were read on a PowerWave XS microplate reader (BIOTEK, Lionheart Technologies Inc, USA) at 450 nm blanking against wells A1 and A2. The blank wells contained GLYCABEN diluent were used as negative controls to ensure that there was no non-specific binding of reagents to the coated plate. A standard curve was plotted, using the KC4 software provided with the microplate reader to generate a dose-response curve. The average optical density of six wells for the standards was used to generate a standard curve which was used for subsequent assays to determine the concentration of glycated albumin in each sample.

The assay was considered valid if the quality controls values were in range as supplied by the manufacturer. In subsequent assays, one of the dilutions of the stock standard used to generate the standard curve was used as internal quality control to check assay validity. Blanks were included in each assay to ensure that there were no non-specific reactions occurring between reagents. Patient samples tested previously were run with the next assay to confirm assay reproducibility.

2.6 DATA ANALYSIS

Summary statistics were calculated using Microsoft Excel (2007) and SPSS, version 15.0. Data were tested for fit to a normal distribution using the Kolmogorov-Smirnov test as implemented in SPSS. Some variables showed normal or near-normal distributions, but since not all variables were normally distributed, subsequent statistical testing was performed using non-parametric methods, unless otherwise indicated. Since some variables were non-normally distributed, the median and interquartile range (IQR), were used as the preferred indicators of central tendency and spread of data, respectively. Since most published studies summarise data as mean values, these were also presented for ease of comparison, although it is recognised that the mean is not as appropriate for data, which are not normally distributed.

Data were not normalised because they did not conform to a general function which could be applied for normalisation. Patients with type 1 diabetes were compared with their corresponding control groups using a Mann-Whitney U test. All tests were 2-sided. The Indian and African patient populations were compared using the Mann-Whitney U test. The variables that differed significantly were HbA1c (higher in Black than in Indian patients, $p = <0.001$), age (higher in Indian than in Black patients, $p = <0.001$), total albumin (higher in Indian than in Black patients , $p = 0.001$), MA (higher in Black than in Indian patients , $p = 0.011$) and proteinuria (higher in Black than in Indian patients, $p = 0.005$). The Black patients were younger in age and had higher median HbA1c , MA and proteinuria values. Indian patients were older and had longer duration of diabetes and higher total serum albumin values compared to Black patients. These variables indicate that at baseline, there was a substantially significant difference between the Black and Indian patient populations.The control populations were also compared to each other. Only the eGFR using the CG formula was higher in Blacks than in Indian patients ($p = 0.001$). The results indicate that at baseline, there where fewer variables that differed between the Indian and Black control populations indicating that they were substantially similar.

Non-parametric stats were used instead of ANOVA because data were not normally distributed. Sequential Mann-Whitney tests were used in preference to a non-parametric ANOVA (Kruskal-Wallis H test) because the Kruskal-Wallis test is weaker than a parametric ANOVA and because pair-wise testing is still required to identify which groups differ significantly if the Kruskal-Wallis test identifies and overall significant difference.

Bivariate correlations were performed in SPSS using Spearman correlation co-efficient. Since correlations could be performed only at each time point individually, generalised estimating equation (GEE) models were performed in SPSS with time specified as a factor in order to take account of relationships among variables over time. The GEE procedure extends the generalized linear regression model to allow for analysis of repeated measurements or other correlated observations (SPSS version 15.0). Models were constructed assuming normal data distribution and linear relationships because the procedure is reported to be robust to some violation of the underlying assumptions (data for a number of variables were normally distributed or near normally distributed) and because software for more complex non-parametric non-linear modelling was not available.

CHAPTER 3

RESULTS

3.1 BASELINE CHARACTERISTICS

At baseline, there was a significant difference between the control and patient groups for all characteristics except age, weight, eGFR using the revised 4-variable MDRD equation, BMI and all blood pressure measurements. Table 5 shows the mean, median and interquartile range (IQR) of clinical variables of the study populations (control and patients). Both ethnic patient populations (Table 6 and 7) had mean fasting plasma glucose (FPG) values above the normal value of 6.4 mmol/L (Black: median 8.0 mmol/L; Indian: median 6.3 mmol/L). Median HbA_{1c} for Black patients was 10.5%, while that for Indian patients was 7.7%, (significant difference, $p < 0.001$). Median serum creatinine for Black patients was 63 µmol/L, while that for Indian patients was 65 µmol/L (significant difference, $p \leq 0.001$). Median duration of diabetes for Black patients was 7.5 years, while that for Indian patients was 17 years (significant difference $p < 0.001$). Mean age of Black patients was 23 years, while that for Indian patients was 31 years (significant difference, $p \leq 0.001$).

Table 5: Characteristics of the total patient population and of the control population at the study outset (baseline)

SD, standard deviation, IQR, interquartile range. NS, not significant. NA, not applicable.
CG – Cockcroft-Gault equation . Cockcroft and Gault, 1976.
4 variable MDRD – Modification of Diet in Renal Disease equation. Levey *et al.* (1999).
Revised 4-v MDRD – Modification of Diet in Renal Disease without ethnicity factor. van Deventer *et al.* (2008).
Reference range – National Health Laboratory Services, Department of Chemical Pathology, Inkosi Albert Luthuli Central Hospital Kwazulu Natal. 2005

Characteristic	Reference range	Statistic	Patients	Controls	p value, difference between patients and controls
n	-	-	83	73	NA
Age (years)	-	Mean ± SD Median IQR	27 ±10 27 14	28 ± 8.0 26 10	NS
Total serum albumin (mg/mL)	34 – 48 (mg/mL)	Mean ± SD Median IQR	40 ± 5 41 7	43 ± 4 42 6	<0.05
Percentage glycated albumin (% GA)	-	Mean ± SD Median IQR	5.8 ± 2.8 5.2 2.8	3.2 ± 1.9 2.6 1.2	<0.001
Fasting plasma glucose (FPG) (mmol/L)	3.1 – 6.4 (mmol/L)	Mean ± SD Median IQR	8.1 ± 4.8 7.3 6	- - -	NA
Random plasma glucose (mmol/L)	3.3 – 7.8 (mmol/L)	Mean ± SD Median IQR	- - -	4.7 ± 0.6 4.7 0.7	N/A
Weight (Kg)	-	Mean ± SD Median IQR	64 ± 13 64 18	64 ± 13 62 15	NS
Serum creatinine (μmol/L)	0 – 106 (μmol/L)	Mean ± SD Median IQR	67 ± 19 64 23	86 ± 16 85 27	<0.001
Body mass index (BMI) Kg/m^2	-	Mean ± SD Median IQR	25 ± 5 24 7	24 ± 5 23 5	NS
Glycohaemoglobin (HbA_{1c})(%)	4.8 – 6.0 %	Mean ± SD Median IQR	9.8 ± 2.6 9.1 3.9	- - -	NA
Pulse (beats per minute)	-	Mean ± SD Median IQR	87 ± 14 85 20	79 ± 9 80 12	<0.001
Urinary type IV collagen (μg/mL)	-	Mean ± SD Median IQR	0.024 ± 0.032 0.010 0.015	0.009 ± 0.014 0.0061 0.0021	<0.001
Plasma fibronectin (mg/mL)	-	Mean ± SD Median IQR	0.37 ± 0.17 0.31 0.16	0.40 ± 0.14 0.38 0.19	<0.05
Isotope glomerular filtration rate (GFR) ($mL/min/1.73m^2$)	-	Mean ± SD Median IQR	103 ± 22 102 39	- - -	NA
Estimated GFR (CG) ($mL/min/1.73\ m^2$)	-	Mean ± SD Median	130 ± 38 125	102 ± 28 99	<0.001

		IQR	44	43	
Estimated GFR 4-variable MDRD(4-v MDRD) (mL/min/1.73 m^2)	-	Mean ± SD Median IQR	144 ± 46 138 50	93 ± 24 89 30	<0.001
Revised 4-variable MDRD (without ethnic factor) (mL/min/1.73 m^2)	-	Mean ± SD Median IQR	144 ± 41 138 50	93 ± 22 89 30	NS
Microalbuminuria (mg/mmol creatinine)	< 3.5 (mg/mmol creatinine)	Mean ± SD Median IQR	6.7 ± 21 0.5 3.2	7.0 ± 21 1.3 3.2	<0.05
Proteinuria (mg/mL)	< 300 (mg/mL)	Mean ± SD Median IQR	1629 ± 4287 300 700	- - -	NA
Blood pressure Supine Systolic (mmHg)	-	Mean ± SD Median IQR	125 ± 18 122 21	118 ± 13 120 15	NS
Blood pressure Supine Diastolic (mmHg)	-	Mean ± SD Median IQR	78 ± 10 80 14	77 ± 10 80 10	NS
Blood Pressure Erect Systolic (mmHg)	-	Mean ± SD Median IQR	125 ± 17 120 23	121 ± 14 120 20	NS
Blood pressure Erect Diastolic (mmHg)	-	Mean ± SD Median IQR	79 ± 10 80 15	78 ± 10 80 18	NS

Table 6: Characteristics of the Black patient population and of control population at the study outset (baseline)

SD, standard deviation, IQR, interquartile range. NS, not significant. NA, not applicable.
CG – Cockcroft-Gault equation . Cockcroft and Gault, 1976.
4 variable MDRD – Modification of Diet in Renal Disease equation. Levey *et al.* (1999).
Revised 4-v MDRD – Modification of Diet in Renal Disease without ethnicity factor. van Deventer *et al.* (2008).
Reference range – National Health Laboratory Services, Department of Chemical Pathology, Inkosi Albert Luthuli Central Hospital, Kwazulu Natal, 2005.

Characteristic	Reference range	Statistic	Black Patients	Controls	p value, difference between patients and controls
n	-	-	54	45	NA
Age (years)	-	Mean ± SD Median IQR	25 ± 8.5 23 10	26 ± 5.5 25 7	NS
Total serum albumin (mg/mL)	34 – 48 (mg/mL)	Mean ± SD Median IQR	41 ± 5 41 7	40 ± 4.7 40 7	< 0.001
Percentage glycated albumin (% GA)	-	Mean ± SD Median IQR	5.8 ± 2.6 5.2 2.6	2.8 ± 1.3 2.5 1.3	<0.001
Fasting glucose (mmol/L)	3.1 – 6.4 (mmol/L)	Mean ± SD Median IQR	8.4 ± 4.8 8.0 5.3	- - -	NA
Random plasma glucose (mmol/L)	3.3 – 7.8 (mmol/L)	Mean ± SD Median IQR	- - -	4.66 ± 0.59 4.7 0.8	N/A
Weight (Kg)	-	Mean ± SD Median IQR	64 ± 3 64 18	67 ± 13 62 14	NS
Serum creatinine (μmol/L)	0 – 106 (μmol/L)	Mean ± SD Median IQR	68 ± 22 63 25	84 ± 16 83 29	< 0.001
Body mass index (BMI) Kg/m^2	-	Mean ± SD Median IQR	25 ± 4 24 6	25 ± 5 25 7	NS
Glycohaemoglobin (HbA_{1c})(%)	4.8 – 6 %	Mean ± SD Median IQR	10.8 ± 7.6 10.5 4.3	- - -	NA
Pulse (beats per minute)	-	Mean ± SD Median IQR	87 ± 14 84 20	78 ± 9 80 16	<0.05
Urinary type IV collagen (μg/mL)	-	Mean ± SD Median IQR	0.0291 ± 0.036 0.0106 0.0036	0.011 ± 0.018 0.0062 0.0026	<0.001
Plasma fibronectin (mg/mL)	-	Mean ± SD Median IQR	0.38 ± 0.17 0.32 0.18	0.42 ± 0.14 0.40 0.21	<0.05
Glomerular filtration rate (GFR) (mL/min)	-	Mean ± SD Median IQR	104 ± 22 108 41	- - -	
Estimated GFR (CG) (mL/min)	-	Mean ± SD Median IQR	130 ± 34 129 41	110 ± 28 113 50	<0.05

Estimated GFR 4-variable MDRD ($mL/min/1.73\ m^2$)	-	Mean ± SD Median IQR	149 ± 46 140 58	97 ± 24 94 33	<0.05
Revised 4-variable MDRD without ethnic factor of 1.212 ($mL/min/1.73\ m^2$)	-	Mean ± SD Median IQR	123 ± 38 116 48	80 ± 20 78 26	NS
Microalbuminuria (mg/mmol creatinine)	< 3.5 (mg/mmol creatinine)	Mean ± SD Median IQR	10 ± 28 0.6 4.2	7.3 ± 23 1.3 2.6	<0.05
Proteinuria (mg/mL)	< 300 (mg/mL)	Mean ± SD Median IQR	1956 ± 4897 300 700	- - -	NA
Blood pressure Supine Systolic (mmHg)	-	Mean ± SD Median IQR	126 ± 18 124 21	118 ± 14 120 18	NS
Blood pressure Supine Diastolic (mmHg)	-	Mean ± SD Median IQR	79 ± 10 80 15	79 ± 10 80 15	NS
Blood pressure Erect Systolic (mmHg)	-	Mean ± SD Median IQR	126 ± 17 124 23	122 ± 14 120 20	NS
Blood pressure Erect Diastolic (mmHg)	-	Mean ± SD Median IQR	80 ± 11 80 18	78 ± 11 80 13	NS

Table 7: Characteristics of the Indian patient population and of control population at the study outset (baseline)

SD, standard deviation, IQR, interquartile range. NS, not significant. NA, not applicable.
CG – Cockcroft-Gault equation . Cockcroft and Gault, 1976.
4 variable MDRD – Modification of Diet in Renal Disease equation. Levey *et al.* (1999).
Revised 4-v MDRD – Modification of Diet in Renal Disease without ethnicity factor. van Deventer *et al.* (2008).
Reference range – National Health Laboratory Services, Department of Chemical Pathology, Inkosi Albert Luthuli Central Hospital Kwazulu Natal, 2005.

Characteristic	Reference range	Statistic	Indian Patients	Controls	p value, difference between patients and controls
n	-		29	28	NA
Age (years)	-	Mean ± SD Median IQR	32 ± 11 31 13	31 ± 10 29 16	NS
Total serum albumin (mg/mL)	34 – 48 (mg/mL)	Mean ± SD Median IQR	42 ± 4 42 6	42 ± 4	
Percentage glycated albumin (% GA)	-	Mean ± SD Median IQR	5.9 ± 3.3 5.2 2.9	3.7 ± 2.5 2.8 1.1	<0.05
Fasting glucose (mmol/L)	3.1 – 6.4 (mmol/L)	Mean ± SD Median IQR	7.5 ± 4.9 6.3 7.3	- - -	NA
Random plasma glucose (mmol/L)	3.3 – 7.8 (mmol/L)	Mean ± SD Median IQR	- - -	4.82 ± 0.64 4.8 0.6	N/A
Weight (Kg)	-	Mean ± SD Median IQR	63 ± 13 62 19	61 ± 14 61 22	NS
Serum creatinine (µmol/L)	0 – 106 (µmol/L)	Mean ± SD Median IQR	65 ± 13 64 18	90 ± 16 91 25	< 0.001
Body mass index (BMI) Kg/m^2	-	Mean ± SD Median IQR	24 ± 5 23 8	23 ± 5 22 7	NS
Glycohaemoglobin (HbA_{1c})(%)	4.8 – 6.0 %	Mean ± SD Median IQR	8.1 ± 1.4 7.7 1.5	- - -	NA
Pulse (beats per minute)	-	Mean ± SD Median IQR	87 ± 14 85 22	81 ± 8 80 12	<0.05
Urinary type IV collagen (µg/mL)	-	Mean ± SD Median IQR	0.0157 ± 0.0195 0.0088 0.0097	0.0062 ± 0.0015 0.0061 0.0015	<0.05
Plasma fibronectin (mg/mL)	-	Mean ± SD Median IQR	0.35 ± 17 0.29 0.12	0.38 ± 13 0.35 0.15	<0.05
Glomerular filtration rate (GFR) (mL/min)	-	Mean ± SD Median IQR	102 ± 22 101 31	- - -	NA
Estimated GFR (CG) (mL/min)	-	Mean ± SD Median IQR	130 ± 45 123 44	88 ± 23 82 28	<0.05

Estimated GFR 4-variable MDRD ($mL/min/1.73\ m^2$)	-	Mean ± SD Median IQR	138 ± 32 128 50	86 ± 18 82 27	<0.05
Revised 4-variable MDRD without ethnic factor of 1.212 ($mL/min/1.73\ m^2$)	-	Mean ± SD Median IQR	114 ± 26 106 41	71 ± 15 67 22	NS
Microalbuminuria (mg/mmol creatinine)	< 3.5 (mg/mmol creatinine)	Mean ± SD Median IQR	2.5 ± 5.1 0.4 1.7	6.6 ±18 1.3 4.2	NS
Proteinuria (mg/mL)	< 300 (mg/mL)	Mean ± SD Median IQR	580 ± 383 300 700	- - -	NA
Blood pressure Supine Systolic (mmHg)	-	Mean ± SD Median IQR	123 ± 17 121 20	118 ± 12 118 14	NS
Blood pressure Supine Diastolic (mmHg)	-	Mean ± SD Median IQR	77 ± 8 77 12	75 ± 10 75 10	NS
Blood pressure Erect Systolic (mmHg)	-	Mean ± SD Median IQR	122 ± 18 120 23	119 ± 14 115 20	
Blood pressure Erect Diastolic (mmHg)	-	Mean ± SD Median IQR	77 ± 7 79 12	78 ± 10 80 20	

3.2 COMPARISON OF VARIABLES RELATING TO DIABETIC NEPHROPATHY

The summary data over time for each variable which relates or is postulated to relate, directly to DN are presented in the following sections. Data are presented for both patient and control groups except for those characteristics which were measured only in the patient group. Isotope GFR, FPG and HbA_{1c} were not measured in the control group due to ethical, logistical and cost factors. Proteinuria was not measured in controls, but MA was. Patients were monitored at baseline, and at six months, one-year and two-year follow-up. Control subjects were monitored only at baseline and 6 months, also for ethical, logistical and cost reasons.

The Box and whisker plots are a visual representation of the distribution of data. The box plot gives the bounds for the data set and the IQR shows the measure of variability. The box plot cannot be used alone as an indicator of the shape of the distribution, for this a histogram, dotplot or stem plot can be used. The value of 1.5 times the IQR is used for convenience to identify those data points outside the IQR and these are identified as outliers. Furthermore, if data are normally distributed (the distribution on which the parameters of the box-plot are based), then the outer limits of the median ± 1.5 IQR incorporate 99% of the data points.

In the following figures, median values are presented as solid lines; the interquartile range (IQR) (25^{th} and 75^{th} percentile) is represented as coloured bar; and the whisker indicates 1.5 times the IQR for the visit. Outliers, defined as values that lie more than1.5 times above or below the IQR, are shown as circles or asterisks. Routine measures of glycaemia (FPG and HbA_{1c}) are presented first. This is followed by the isotope GFR used to assess kidney function. The measured GFR was compared to estimated GFR (eGFR) using the 4-v MDRD, revised 4-v MDRD and CG equations. The levels of proteinuria in control and patient groups were assessed to identify patients with established glomerular pathology. Levels of microalbumin were used to identify patients at risk of progressing to proteinuria. Finally, the new markers of renal function (urinary type IV collagen and plasma fibronectin and serum glycated albumin) were assessed in their ability to identify declining renal function and so identify patients at increased risk of DN.

3.2.1 Fasting plasma glucose (FPG) and haemoglobin A_{1c} (HbA_{1c})

The measurement of FPG and of HbA_{1c} is routinely used to identify patients with hyperglycaemia. Diabetes is diagnosed if FPG is 7.0 mmol/L or higher and confirmed on one subsequent occasion. Levels of HbA_{1c} are elevated when blood glucose is elevated. Glycated haemoglobin (HbA_{1c}) is routinely measured to give an estimate of the plasma glucose levels over the past 4 months and should ideally be below 7% (American Diabetes Association 2004). The results of the fasting glucose and HbA_{1c} measured at each visit are presented below.

3.2.1.1 Fasting plasma glucose for Black patients

At baseline, median FPG was 8.0 mmol/L. Median FPG values across visits were higher than the normal range (3.1 to 6.4 mmol/L) used by the laboratory performing the testing (Figure 9). Median FPG values remained fairly constant across visits.

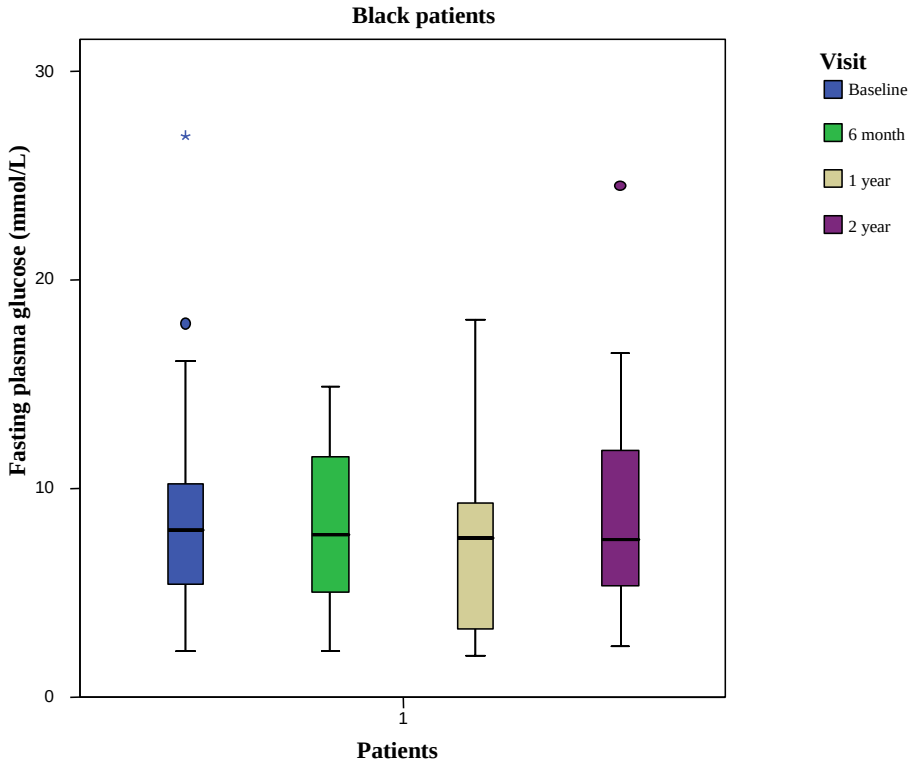

Figure 9 Box plot of fasting glucose in Black patients from baseline to two year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.1.2 Haemoglobin A_{1c} for Black patients

At baseline the median HbA_{1c} was 10.5%. Median HbA_{1c} values across visits remained relatively constant and were above the normal range (4.8 to 6.0%) used by the laboratory performing the testing (Figure 10). Elevated FPG corresponded with elevated HbA_{1c} values across visits (Figure 9).

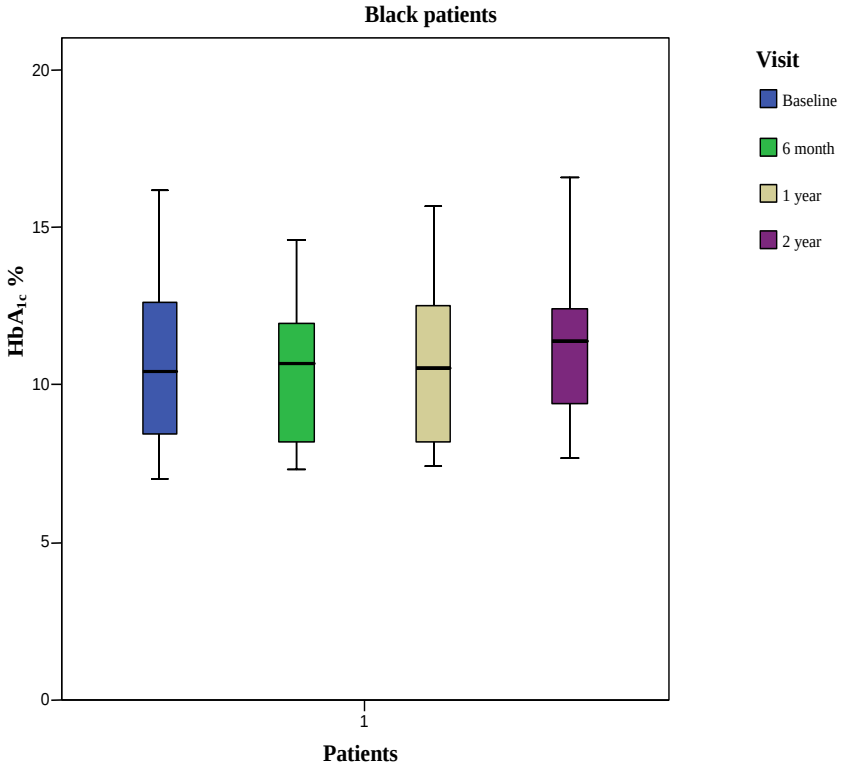

Figure 10:Box plot of percentage HbA_{1c} in Black patients from baseline to two year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.1.3 Fasting plasma glucose for Indian patients

At baseline, median FPG was 6.3 mmol/L, lower than for Black patients. The median FPG values across visits were above the normal range (3.1 to 6.4 mmol/L) used by the laboratory performing the testing (Figure 11). There was a trend of increasing FPG from six months to two-year follow. Median FPG increased significantly relative to baseline over the two-year follow-up period ($p \leq 0.05$).

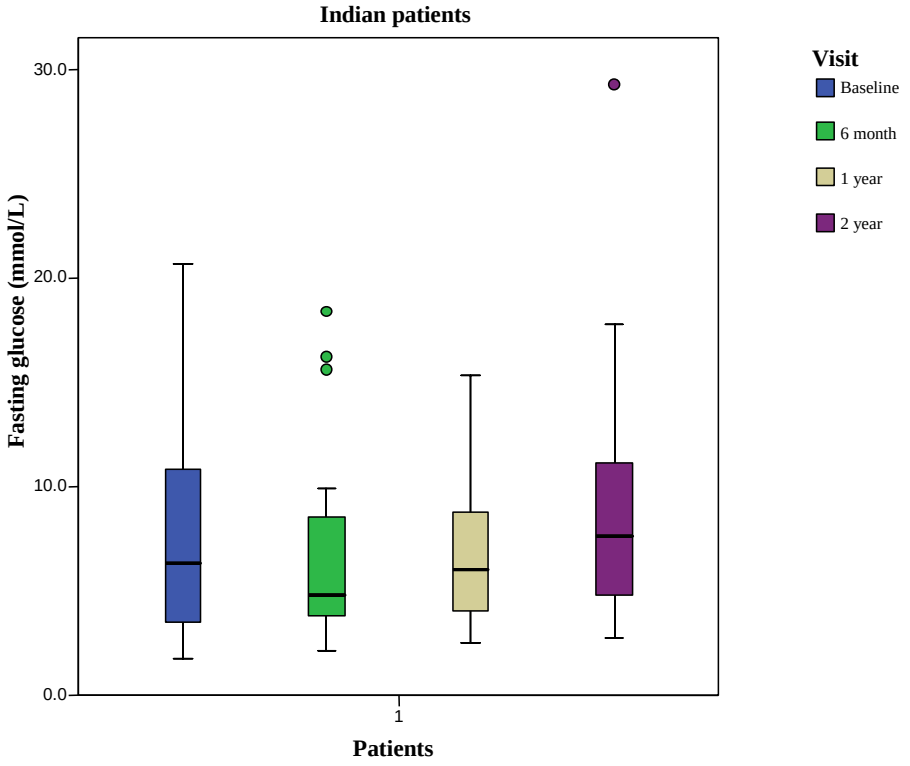

Figure 11: Box plot of FPG in Indian patients from baseline to two-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.1.4 Haemoglobin A_{1C} for Indian patients

At baseline the median HbA_{1c} was 7.7%, lower than for Black patients. The median HbA_{1c} values across visits were higher than the normal range (4.8 to 6.0%) used by the laboratory performing the testing (Figure 12). There was a trend of increasing HbA_{1C} with time, a similar trend as was seen with FPG (Figure 11). Median HbA_{1c} increased significantly from baseline to two-year follow-up ($p \leq 0.001$).

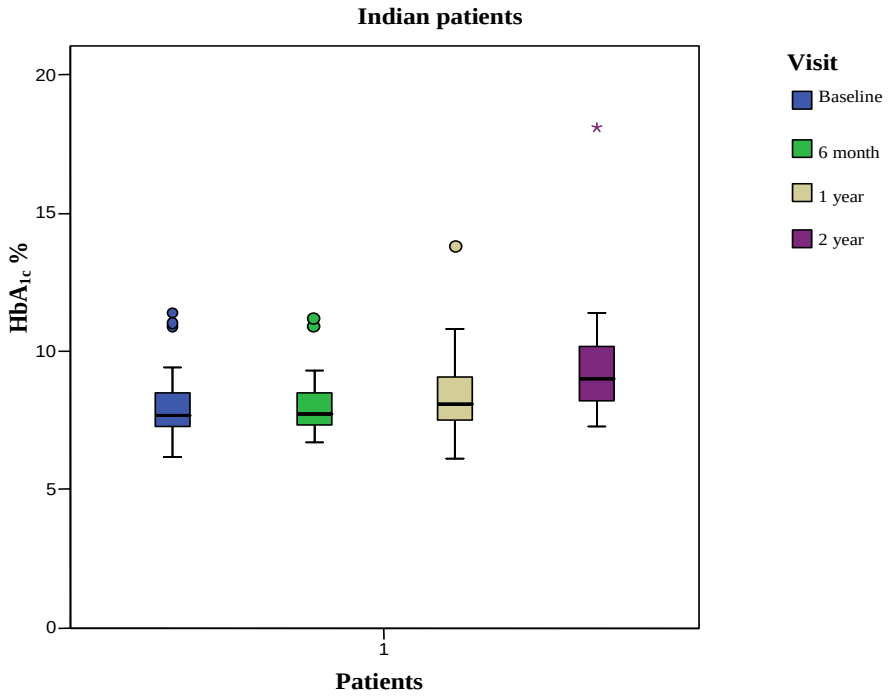

Figure 12. Box plot of HbA1c percentage in Indian patients from baseline to 2-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values, which lie 1.5 times above or below the IQR.

3.2.2 Isotope glomerular filtration rate (GFR)

The gold standard of measuring kidney function is using an isotopic method of determining GFR. Glomerular filtration values below 90 mL/min/1.73 m^2 are associated with decreasing kidney function and are staged as indicated in Chapter 1. The goal of performing GFR measurements was to identify patients with CKD. The GFR results for patients tested are presented in Figures 13 and 14 below.

3.2.2.1 Isotope GFR for Black patients

At baseline, median GFR was 108 mL/min/1.73 m^2 . This value was below the normal value (males: 130 mL/min/1.73m2; females: 120 mL/min/1.73 m^2) used by the laboratory performing the testing. There was a trend of abnormal GFR (< 90 mL/min/1.73 m^2 , CKD stage 2) across visits (Figure 13). Median isotope GFR values at two-year follow-up (104 mL/min/1.73 m^2) were lower than at baseline (108 mL/min/1.73 m^2) but this did not reach statistical significance.

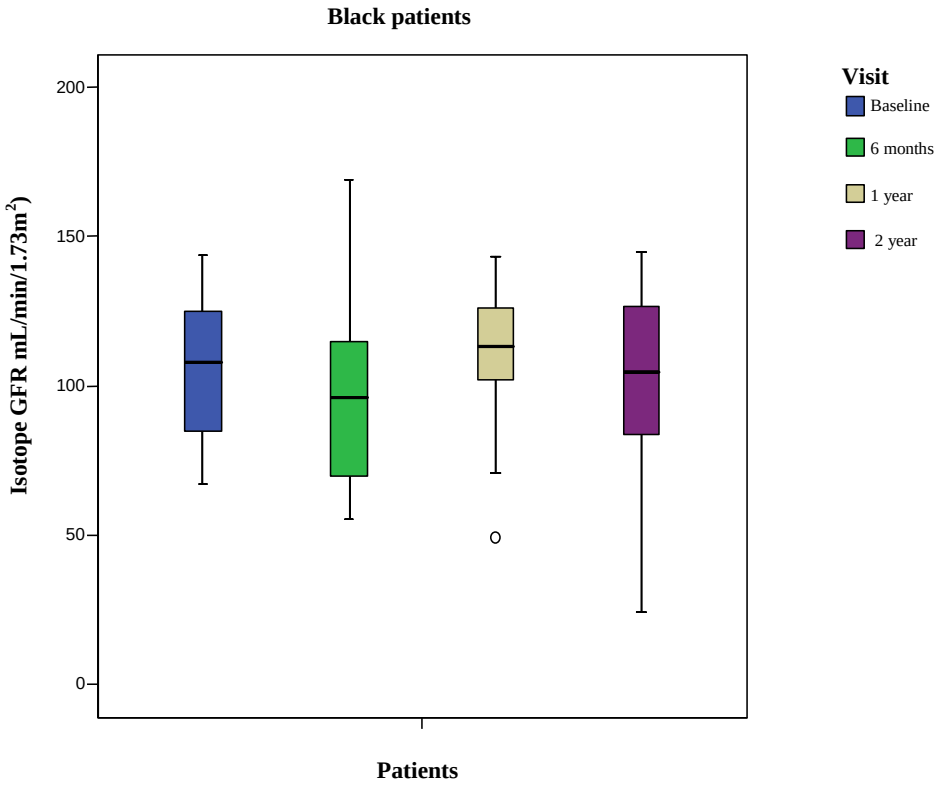

Figure 13. Box plot of isotope GFR in Black patients from baseline to 2-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.2.2 Isotope GFR for Indian patients

At baseline, median GFR was 101 mL/min/1.73 m^2, lower than for Black patients. Abnormal GFR were observed across visits (< 90 mL/min/1.73 m^2 , CKD stage 2) (Figure 14). At 2-year follow-up the median GFR values were comparable to those at baseline.

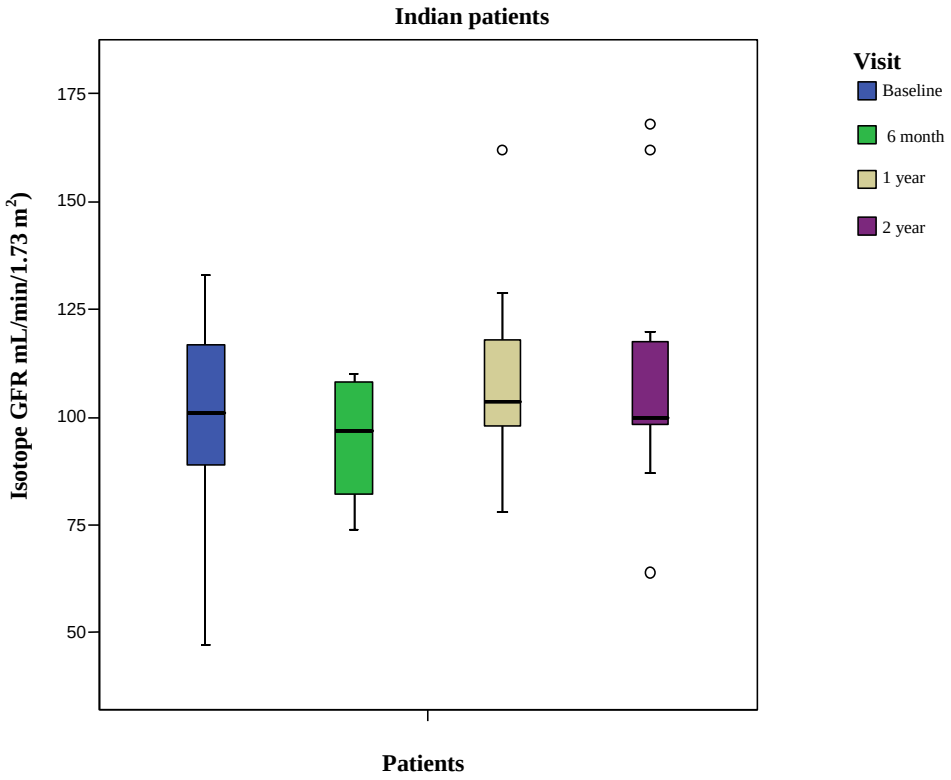

Figure 14. Box plot of isotope GFR in Indian patients from baseline to 2-year. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.3 Estimated glomerular filtration rate (eGFR)

The use of an estimated GFR (eGFR) value during routine renal function assessments provides clinicians with a cost-effective means of monitoring the efficiency of kidney function in diabetic patients. The results of eGFR calculated with the 4-variable MDRD equation (Figure 15 and 16), revised 4-variable MDRD equation (Figure 17 and 18) and Cockcroft-Gault (Figure 19 and 20) equation for patients at each visit are presented below.

3.2.3.1 Estimated GFR using the 4-variable MDRD equation for Black patients

Estimated GFR, using the 4-v MDRD equation, was significantly different ($p \leq 0.001$) between patients and control subjects at both baseline and 6-month follow-up. There was a trend of decreasing eGFR values in patients from 6-months to 2-year follow-up (Figure 15). At baseline, median eGFR values for Black patients was 140 mL/min/1.73 m^2. This value was higher than the median value of 94 mL/min/1.73 m^2 for controls. The median eGFR values (4-v MDRD equation) across visits were within 30% of the isotope GFR (baseline: 140 mL/min/1.73 m^2 *vs* 108 mL/min/1.73 m^2; 6-month follow-up: 146 mL/min/1.73 m^2 *vs* 96 mL/min/1.73 m^2: 1-year: 133 mL/min/1.73 m^2 vs 113 mL/min/1.73 m^2; 2-year: 124 mL/min/1.73 m^2 vs 105 mL/min/1.73 m^2). There was a significant decrease ($p \leq 0.001$) between the baseline and 2-year eGFR value in patients.

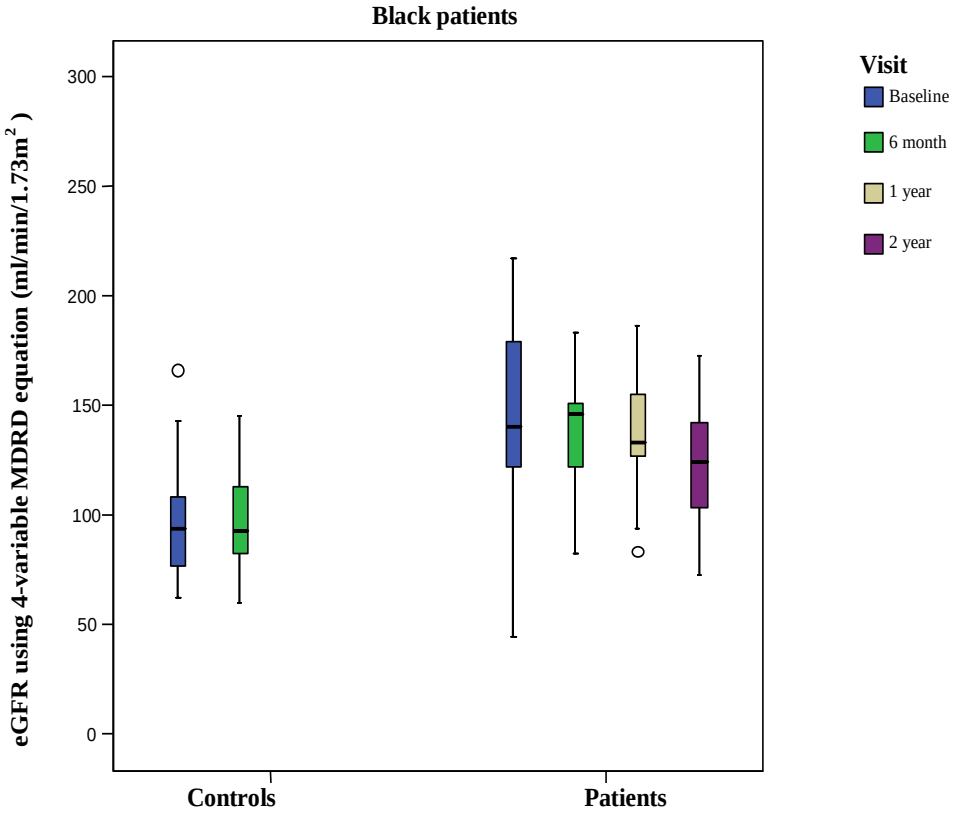

Figure 15. Box plot of eGFR using the 4-v MDRD formula in Black control and patient groups. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values, which lie 1.5 times above or below the IQR.

3.2.3.2 Estimated GFR using the 4-variable MDRD equation for Indian patients

Estimated GFR, calculated using the 4-v MDRD equation was significantly different ($p < 0.001$) between patients and control subjects at both baseline and 6-month follow-up. At baseline, median eGFR values for patients was 128 mL/min/1.73 m^2, compared to 86 mL/min/1.73 m^2 for controls. Median eGFR values at baseline were lower than for Black patients. There was a trend of decreasing eGFR values across visit (Figure 16). Median eGFR values (4-v MDRD) across visits were within 30% of the isotope GFR (baseline: 128 mL/min/1.73 m^2 *vs* 101 mL/min/1.73m^2; 6-month: 126 mL/min/1.73m^2 *vs* 97 mL/min/1.73 m^2; 1-year: 129 mL/min/1.73 m^2 *vs* 104 mL/min/1.73 m^2; 2-year: 116 mL/min/1.73 m^2 *vs* 100 mL/min/1.73 m^2). There was a significant decrease ($p \leq 0.001$) between the baseline and 2-year eGFR value in patients.

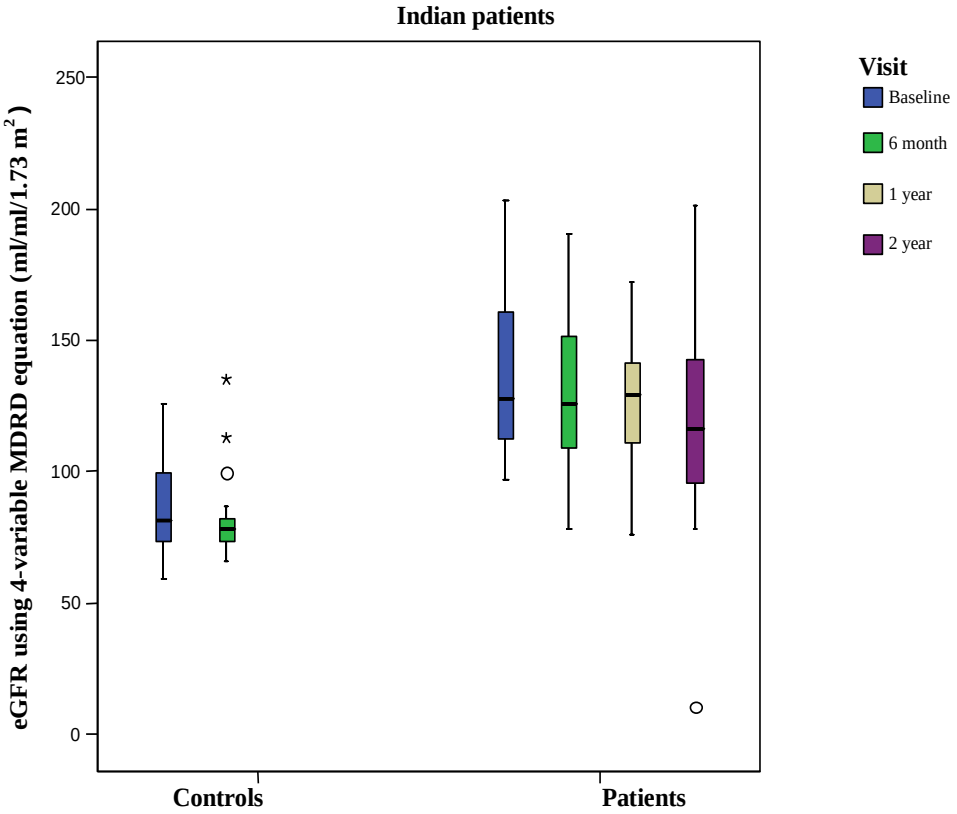

Figure 16. Box plots of estimated GFR, calculated using the 4-variable MDRD equation in Indian control and patient groups from baseline to 2-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.3.3 Estimated GFR using the revised 4-variable MDRD equation without ethnicity factor for Black patients

Estimated GFR, using the revised 4-v MDRD equation was significantly different ($p < 0.001$) between patients and controls at both baseline and 6-month follow-up. At baseline, median eGFR values for patients was 116 mL/min/1.73m^2 compared to 78 mL/min/1.73 m^2 for controls. There was a trend of decreasing eGFR values across visit in patients (Figure 17). The median eGFR values (revised 4-v MDRD equation) across visits were within 30% of the isotope GFR (baseline: 116 mL/min/1.73 m^2 *vs* 108 mL/min/1.73 m^2; 6-month: 120 mL/min/1.73 m^2 *vs* 96 mL/min/1.73 m^2 ; 1-year: 110 mL/min/1.73m^2 *vs* 113 mL/min/1.73m^2; 2-year: 102 mL/min/1.73m^2 *vs* 105 mL/min/1.73m^2). There was a significant decrease ($p \leq 0.001$) between the baseline and 2-year eGFR value in patients.

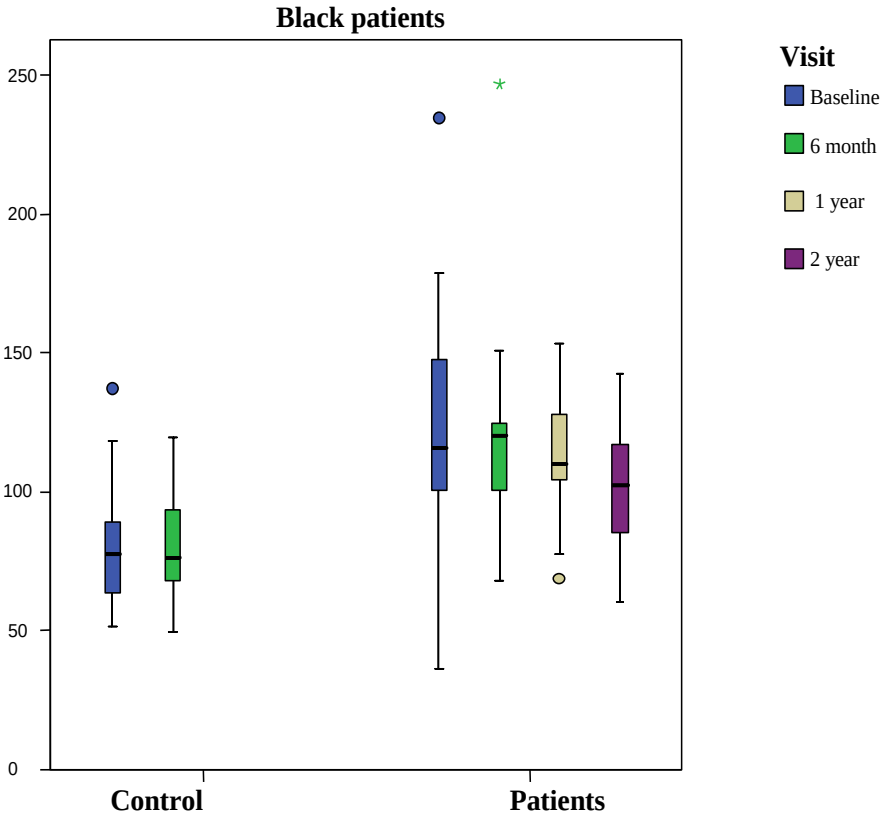

Figure 17. Box plots of eGFR, using the revised 4-variable MDRD equation in Black control and patient groups from baseline to 2-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.3.4 Estimated GFR using the revised 4-variable MDRD equation without ethnicity factor for Indian patients

Estimated GFR, calculated using the revised 4-v MDRD equation was significantly higher ($p < 0.001$) in patients than in control at both baseline and 6-month follow-up. At baseline, median eGFR values for patients was 106 mL/min/1.73 m^2 compared to 67 mL/min/1.73 m^2 for controls, lower than in Black patients. The median value at baseline was lower than in Black patients. There was a trend of decreasing eGFR values across visit (Figure 18). The median eGFR values across visits were within 30% of the isotope GFR (baseline: 128 mL/min/1.73 m^2 *vs* 101 mL/min/1.73 m^2; 6-month: 126 mL/min/1.73 m^2 *vs* 97 mL/min/1.73 m^2; 1-year: 106 mL/min/1.73m^2 *vs* 104 mL/min/1.73 m^2; 2-year: 96 mL/min/1.73 m^2 *vs* 100 mL/min/1.73 m^2). There was a significant decrease ($p \leq 0.001$) between the baseline and 2-year eGFR value in patients.

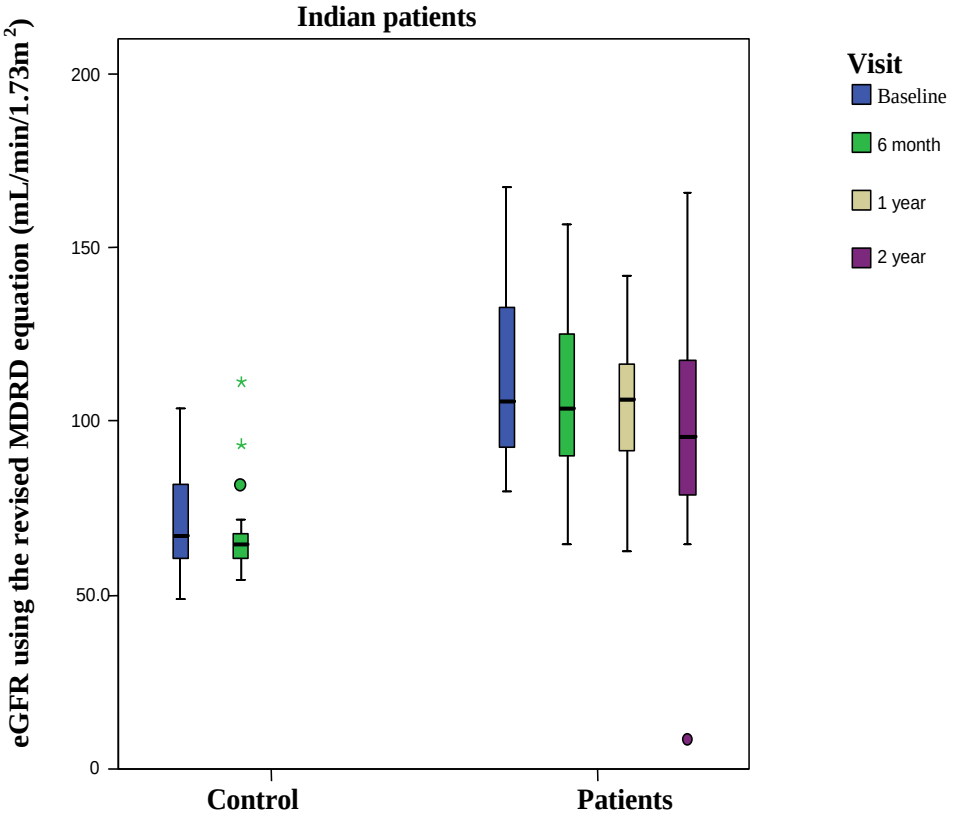

Figure 18. Box plots of eGFR, using the revised 4-variable MDRD equation in Indian control and patient groups from baseline to 2-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.3.5 Estimated GFR using Cockcroft-Gault (CG) equation for Black patients

Estimated GFR, calculated using the CG equation was significantly higher ($p \leq 0.05$) in patients than in controls at both baseline and 6-month follow-up. At baseline, median eGFR values for patients was 128 mL/min/1.73 m^2 compared to 86 mL/min/1.73 m^2 for controls. There was a trend of decreasing eGFR values across visits (Figure 19). The median eGFR values (CG equation) across visits were within 30% of the isotope GFR (baseline: 129 mL/min/1.73m^2 *vs* 109 mL/min/1.73 m^2; 6-month: 131 mL/min/1.73m^2 *vs* 96 mL/min/1.73m^2; 1-year: 123 mL/min/1.73 m^2 vs 113 mL/min/1.73m^2 ; 2-year: 110 mL/min/1.73 m^2 *vs* 105 mL/min/1.73m^2). There was a significant decrease ($p \leq 0.05$) between the baseline and 2-year eGFR value in patients. At end-point, eGFR values in the patients were similar to the baseline values.

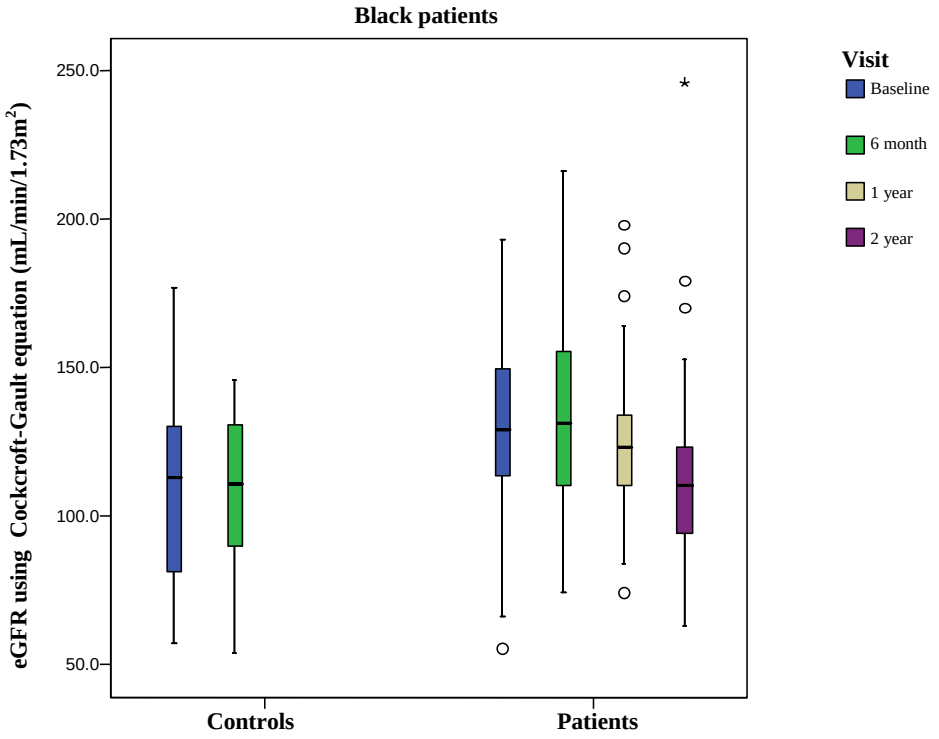

Figure 19. Box plot of eGFR using the Cockcroft-Gault equation in Black control and patient groups. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.3.6 Estimated GFR using the Cockcroft-Gault equation for Indian patients

Estimated GFR, using the CG equation was significantly higher ($p < 0.001$) in patients than in controls at both baseline and 6-month follow-up. At baseline, median eGFR values for patients was 123 mL/min/1.73 m^2 compared to 82 mL/min/1.73 m^2 for controls. At 6-months, median eGFR values for patients was 115 mL/min/1.73 m^2 compared to 82 mL/min/1.73 m^2 for controls. There was a trend of decreasing eGFR values across visits in patients (Figure 20).
The median eGFR values (CG equation) across visits were within 30% of the isotope GFR (baseline: 123 mL/min/1.73m^2 *vs* 101 mL/min/1.73 m^2; 6-month: 115 mL/min/1.73 m^2 *vs* 97 mL/min/1.73 m^2; 1-year: 111 mL/min/1.73 m^2 *vs* 104 mL/min/1.73m^2; 2-year; 115 mL/min/1.73m^2 *vs* 100 mL/min/1.73m^2). Median eGFR values decreased significantly from baseline to two-year follow-up ($p \leq 0.05$).

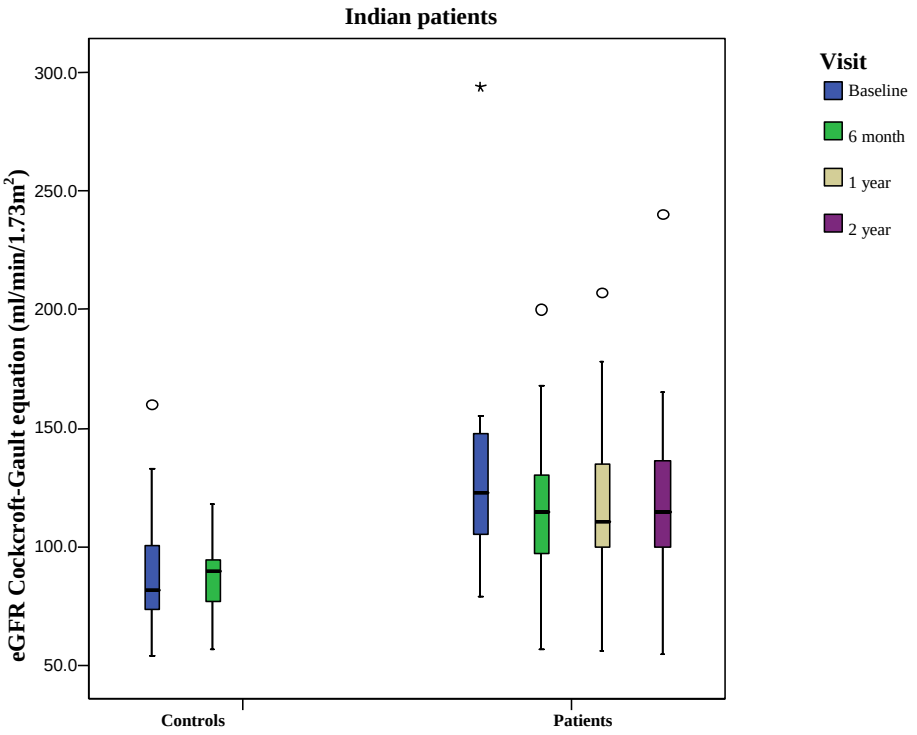

Figure 20. Box plot of eGFR using the Cockcroft-Gault equation in Indian control and patient groups. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.4 Serum creatinine

Routinely, the measurement of serum creatinine is used as a marker of kidney function. Under conditions of compromised kidney function (*e.g.* diabetic nephropathy), serum creatinine values increase. The results of serum creatinine across visits for patients with diabetes are presented below.

3.2.4.1 Serum creatinine in Black patients

At baseline and 6-month follow-up the median serum creatinine value in Black patients was significantly ($p \leq 0.001$) lower than that in the control group (baseline: 63 µmol/L *vs* control: 83 µmol/L; 6-month: 67 µmol/L *vs* control: 86 µmol/L). There was a trend of increasing serum creatinine across visits in patients (Figure 21). The median serum creatinine at 2-year follow-up was significantly higher ($p \leq 0.001$) than that at baseline in the patients (75 µmol/L *vs* 63 µmol/L). The serum creatinine value at 2-year follow-up was comparable to that of the control group.

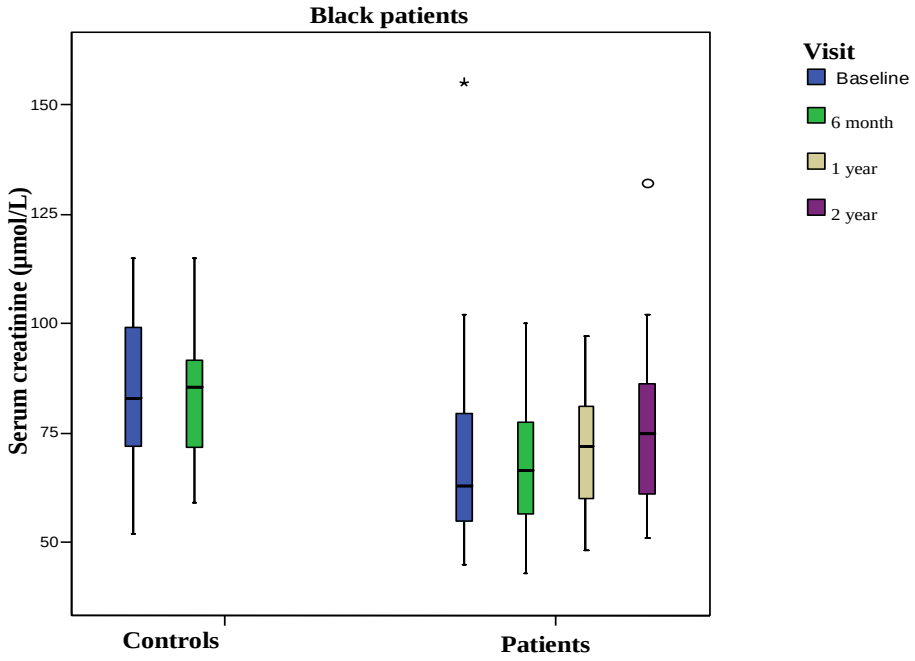

Figure 21. Box plot of serum creatinine in Black control and patient groups. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.4.2 Serum creatinine in Indian patients

At baseline and 6-month follow-up the serum creatinine value in Indian patients was significantly ($p \leq 0.001$) lower than in the control group (baseline: median 65 µmol/L vs control: 91 µmol/L; 6-month: 69 µmol/L vs control: 91 µmol/L), higher than that in Blacks. There was a trend of increasing median serum creatinine across visits in the patients (Figure 22). The serum creatinine in the patients at 2-year follow-up was significantly ($p \leq 0.001$) higher than at baseline (75 µmol/L vs 63 µmol/L).

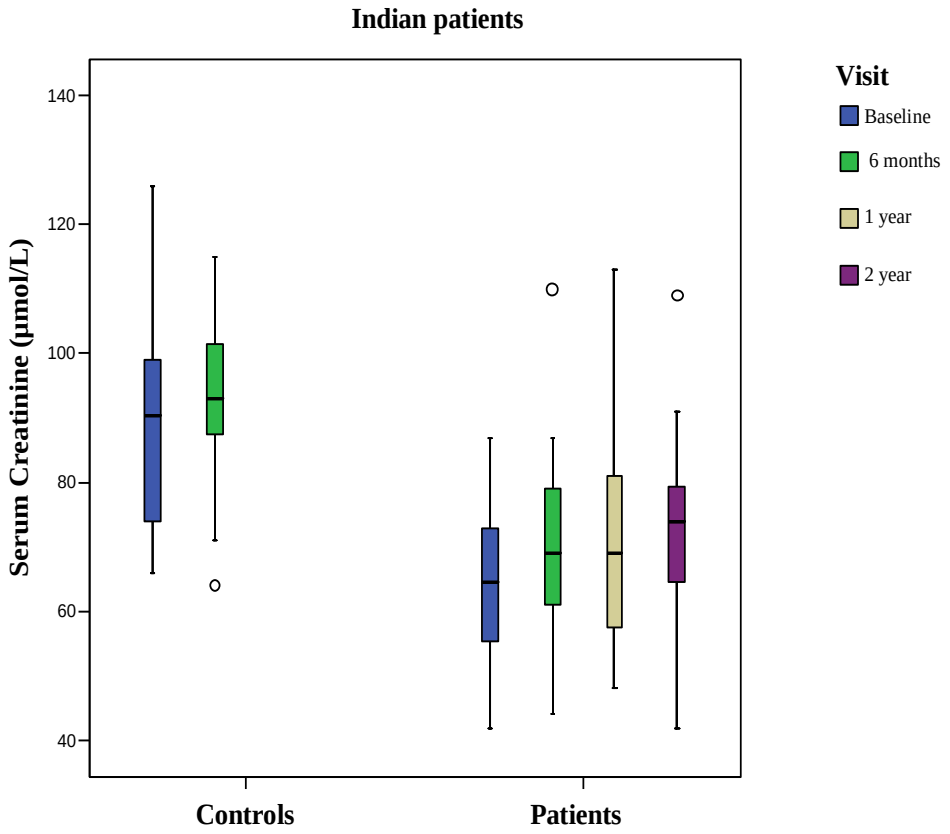

Figure 22. Box plot of serum creatinine in Indian control and patient groups. Bars show median value. Box shows the interquartile range (IQR). Whisker shows the lowest and highest value. Outliers are values which lie 1.5 times above or below the IQR.

3.2.5 Microalbuminuria (MA) and proteinuria

Microalbuminuria is an indicator of declining renal function and incipient DN. Proteinuria is a measure of declining renal function and a risk factor for DN in patients with diabetes.

3.2.5.1 Microalbuminuria in Black patients

At baseline there was no significant difference ($p = 0.087$) in the level of MA between patients and controls. At 6-months follow-up, there was a significant difference ($p \leq 0.05$) in the level of MA, which was higher in controls than in patients. At baseline median MA was 1.2 mg/mmoL in patients. There was a trend of decreasing median MA values from baseline to 1 year follow-up in patients (baseline: 1.2 mg/mmoL; 6-months: 1.0 mg/mmoL; 1-year: 0.45 mg/mmoL) (Figure 23). At 2-year follow-up the median MA was comparable to that at baseline (1.4 mg/mmoL). Microalbuminuria persisted across visits.

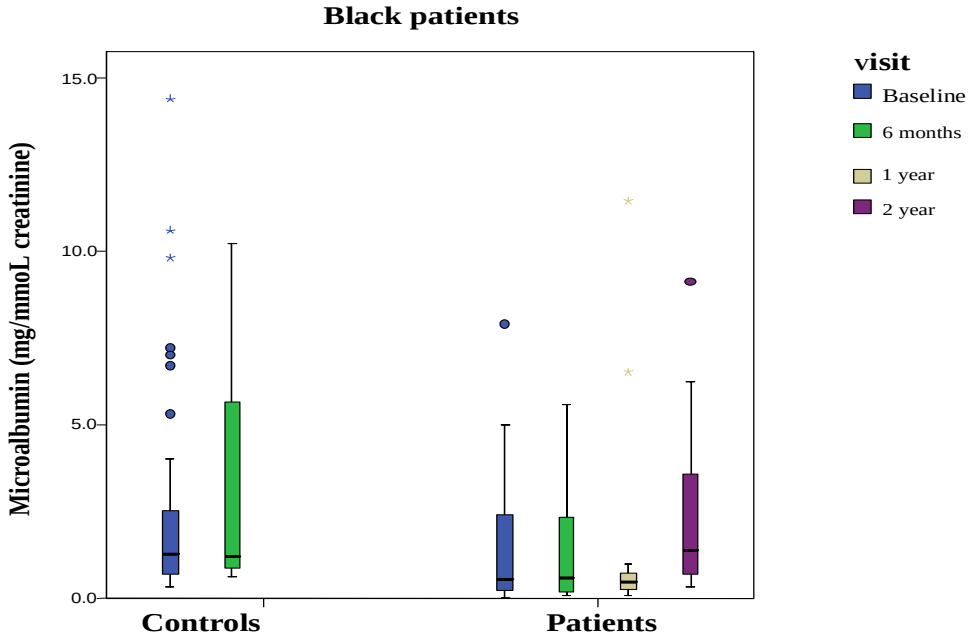

Figure 23. Box plot of microalbuminuria in Black control and patient groups from baseline to 2-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR. Outliers above 30 mg/mmoL creatinine removed (three points in controls and three points in patients).

3.2.5.2 Proteinuria in Black patients

At baseline median, albumin concentration in urine for Black patients was 300 mg/mL. Albuminuria persisted at each visit (Figure 24). Median albumin concentration did not increase significantly relative to baseline over the two-year follow-up period. Of the 16 MA patients at baseline, 44% (7/16) progressed to proteinuria at 2-year follow-up (results not shown). Of the nine proteinuric patients at baseline, 22% (2/9) regressed to MA and 44% (4/9) to < 3.4 mg/mmoL (results not shown).

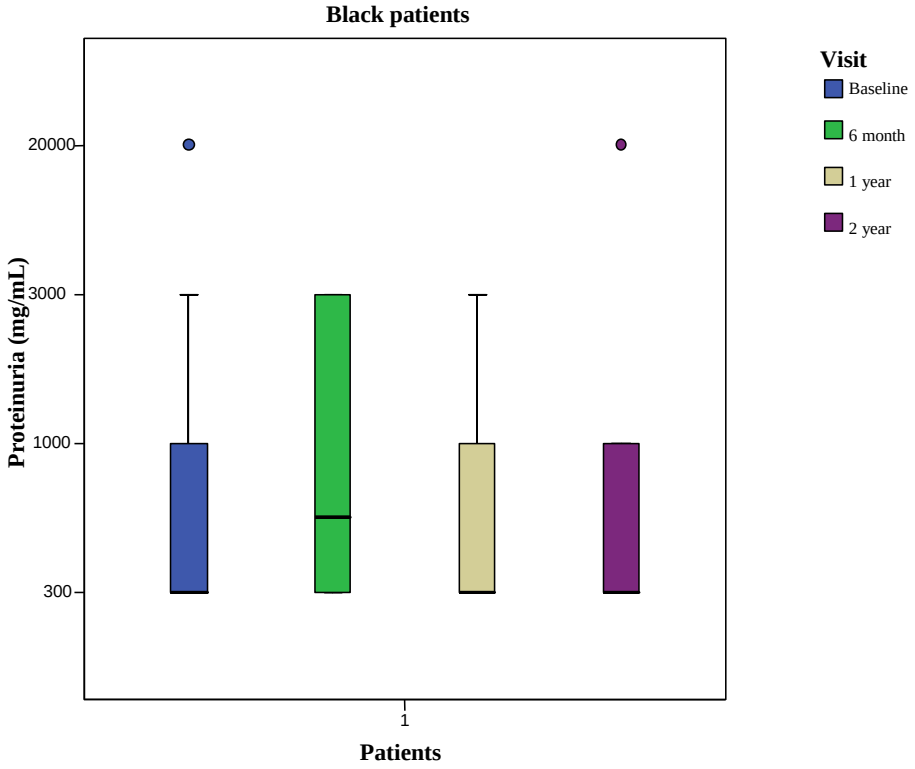

Figure 24. Box plot of proteinuria in Black patients from baseline to 2-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.5.3 Microalbuminuria in Indian patients

There was a significant difference ($p \leq 0.05$) in the level of MA between patients and controls at baseline and 6-months follow-up. At baseline and 6-month follow-up, median MA was higher in the control group compared to the patients (Baseline: controls, 1.3 mg/mmol creatinine vs patients, 1.1 mg/mmol creatinine; 6 months: controls,1.3 mg/mmol creatinine *vs* patients: 1.1 mg/mmol creatinine for control subjects) (Figure 25). Median MA increased significantly ($p \leq 0.05$) from baseline to two year follow-up. High median values in controls may be influenced by a small number of outliers (although influence is less than on mean).

In the patient population ,MA persisted across visits.

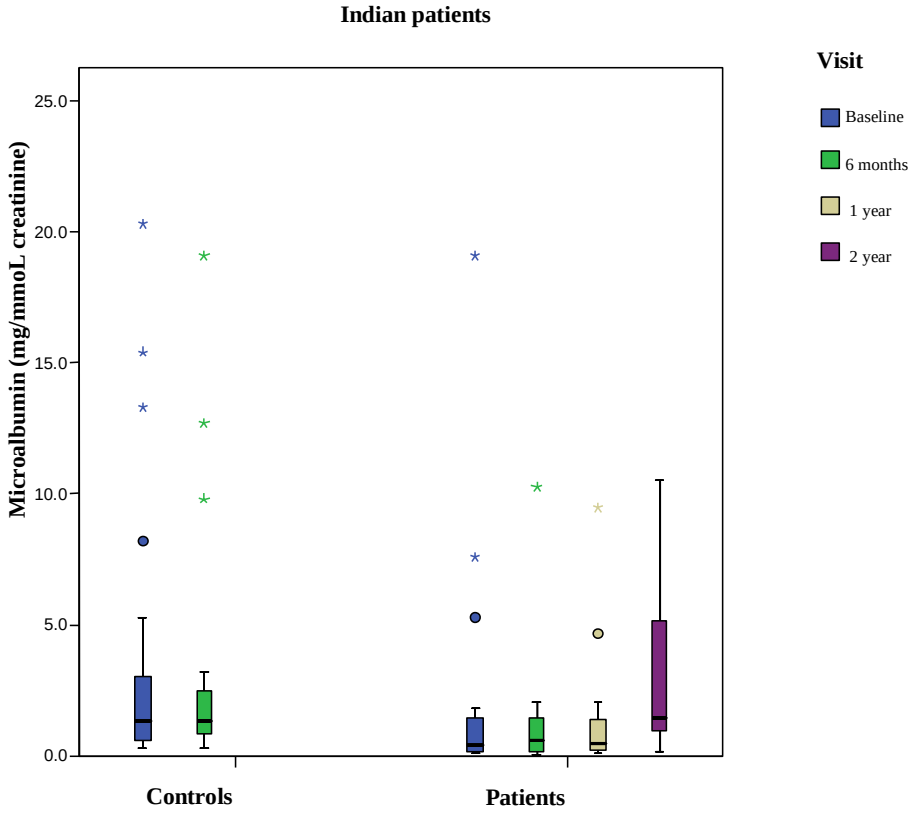

Figure 25. Box plot of Microalbuminuria in Indian control and patient groups from baseline to 2-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.. Outliers above 30 mg/mmoL creatinine removed (one from controls).

3.2.5.4 Proteinuria in Indian Patients

At baseline median albumin concentration in urine for Indian patients was 300 mg/mL. Proteinuria persisted at each visit (Figure 26). Of the four proteinuric patients at baseline, 25% (1/4) regressed to MA and 25% (1/4) to < 3.4 mg/mmoL creatinine (results not shown).

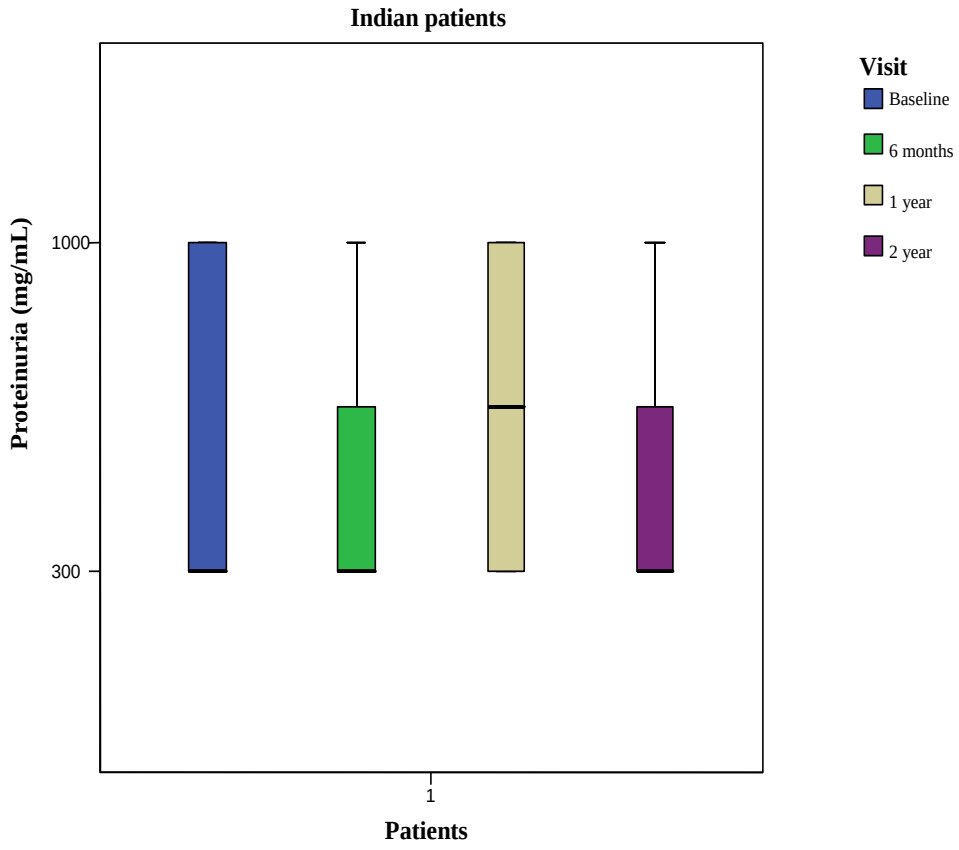

Figure 26. Box plot of proteinuria in Indian patients from baseline to 2-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.6 Percentage glycated albumin (PGA)

Glycated albumin is used as a short- to medium-term marker of hyperglycaemia compared to HbA_{1c} and may be directly involved in pathology of DN .

3.2.6.1 Percentage glycated albumin in Black patients

At baseline, median PGA in Black patients was 5.20%. The PGA concentration at baseline ($p \leq 0.001$) and 6 months ($p \leq 0.05$) was significantly higher in patients than in controls. There was a trend of decreasing median GA values across visits in patients (baseline: median 5.20%; 6 month: 3.68%; 1 year: 1.97%; 2 year: 1.87%) (Figure 27).

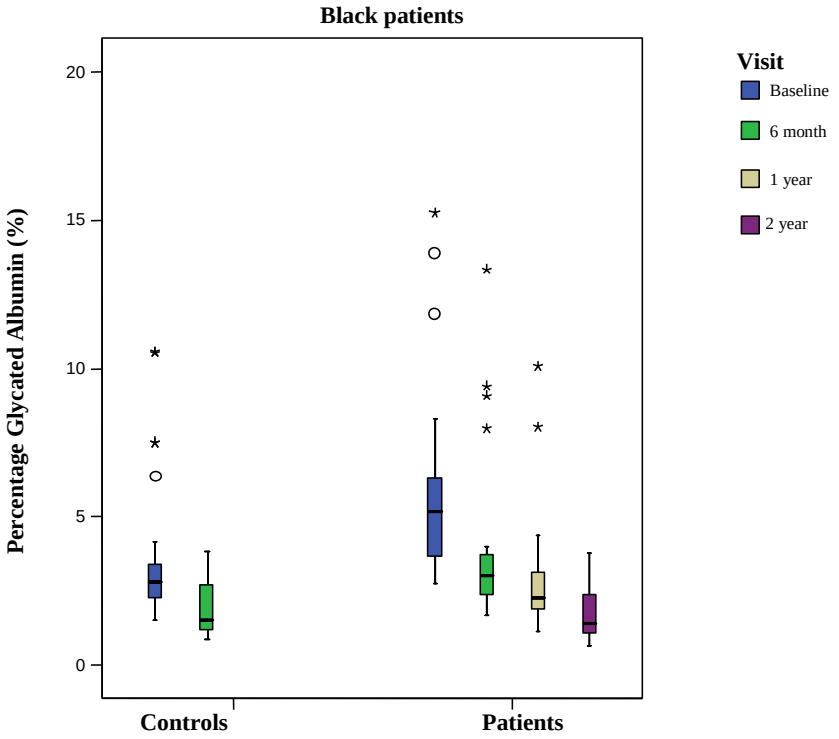

Figure 27. Box plot of percentage glycated albumin (PGA) in Black control and patient groups from baseline to 2-year follow-up. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.6.2 Percentage glycated albumin in Indian patients

At baseline, the median PGA in Indian patients was 5.17%, lower than in Blacks. At baseline and 6 months the PGA was significantly higher ($p \leq 0.05$) in patients than in controls. There was a trend of decreasing median PGA values across visits (baseline: median 5.17%; 6 month: median 3.00%; 1 year: median 2.27%; 2 year: median 1.42) (Figure 28).

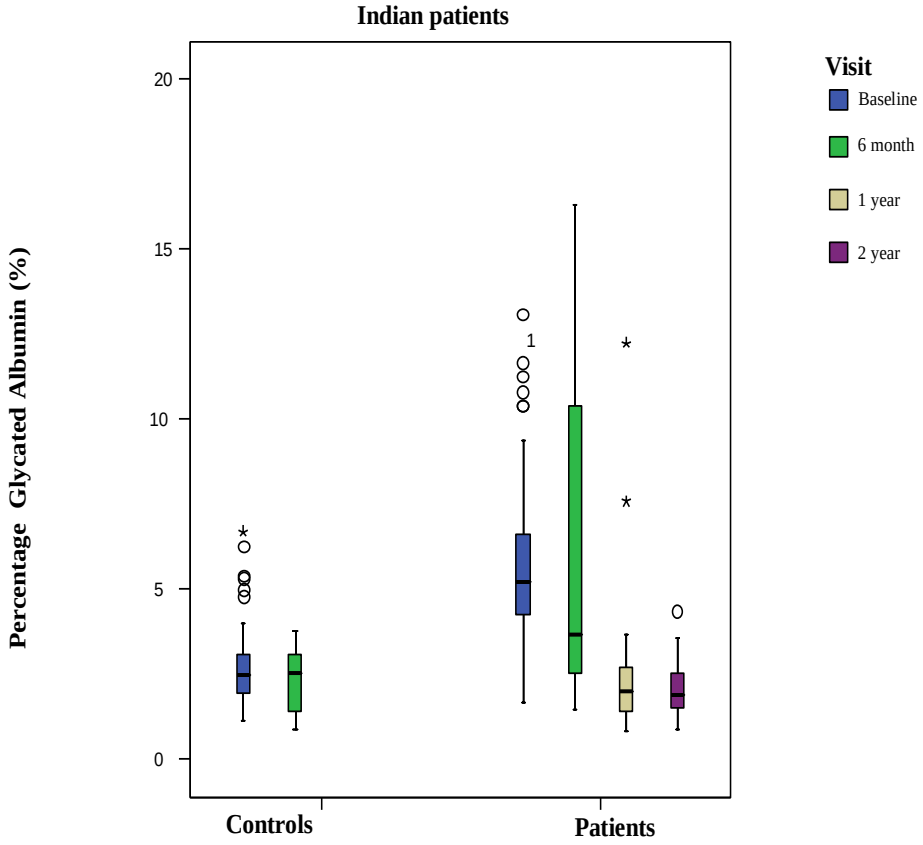

Figure 28. Box plot of percentage glycated albumin (PGA) in Indian control and patient groups. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.7 Urinary type IV collagen

Measurement of type IV collagen in urine has been shown to be a marker of declining kidney function in diabetic patients.

3.2.7.1 Urinary type IV collagen in Black Patients

At baseline and 6-months follow-up, urinary type IV collagen in Black patients was significantly higher ($p \leq 0.001$) than in controls. Median urinary type IV collagen concentration in patients at baseline was 0.0106µg/mL compared to 0.0371 µg/mL at 6-months follow-up. Median urinary collagen IV increased with time, with sudden drop at 2-year follow-up (Figure 29). The median urinary type IV collagen at 2-year follow-up was comparable to that at baseline (baseline: 0.0106 µg/mL *vs* 2-year: 0.0111 µg/mL).

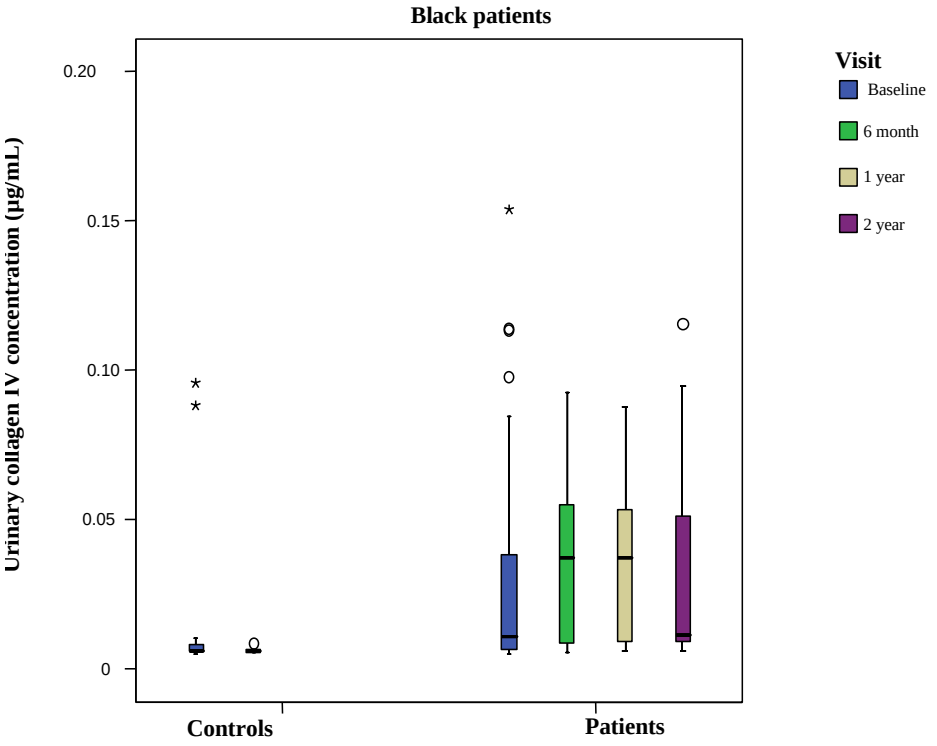

Figure 29. Box plot of urinary collagen IV concentration in Black control and patient groups. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.7.2 Urinary type IV collagen in Indian Patients

Urinary type IV collagen concentration was significantly higher in patients at baseline ($p \leq 0.05$) and 6 months ($p \leq 0.001$) than in controls. In patients, median urinary type IV collagen concentration at baseline was 0.0088 µg/mL compared to 0.0102 µg/mL at 6-months follow-up, lower than in Blacks. Urinary type IV collagen concentration increased with time, with sudden drop at 2-year follow-up (Figure 30). At 2-year follow-up the median urinary type IV collagen value was comparable to that at baseline (baseline: 0.0088 µg/mL *vs* 2-year: 0.0108 µg/mL).

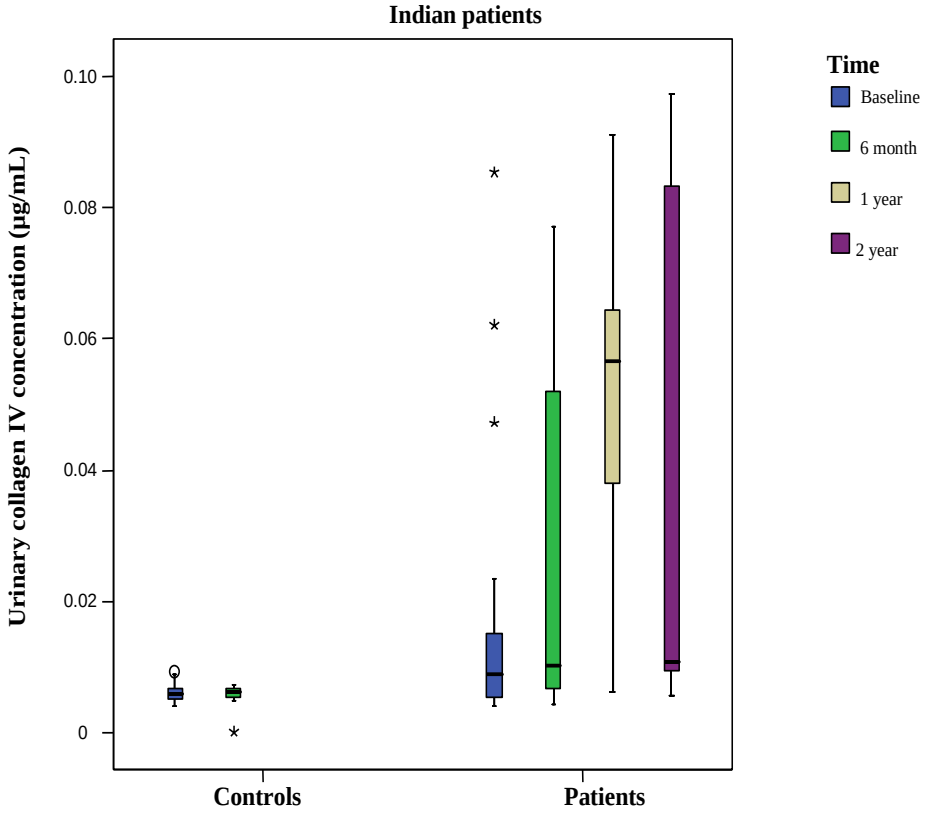

Figure 30. Box plot of urinary collagen IV concentration in Indian control and patient subjects. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.8 Plasma fibronectin (FN)

Fibronectin is a component of the GBM. Increased levels of plasma FN have been proposed as a marker of incipient diabetic nephropathy.

3.2.8.1 Plasma FN in Black patients

At baseline and 6-months follow-up, median plasma FN in patients was significantly lower ($p \leq 0.05$) than in the control population (baseline: 0.32 mg/mL vs control 0.40 mg/ml; 6 months: median 0.37 mg/mL *vs* control 0.59 mg/mL). In the patients, the median plasma FN at baseline and 6-months follow-up was within the normal range of 0.20 to 0.40 mg/mL (Figure 31). At end-point, the median fibronectin concentration in patients (0.54 mg/mL) was higher than the normal range (0.2 to 0.4 mg/mL).

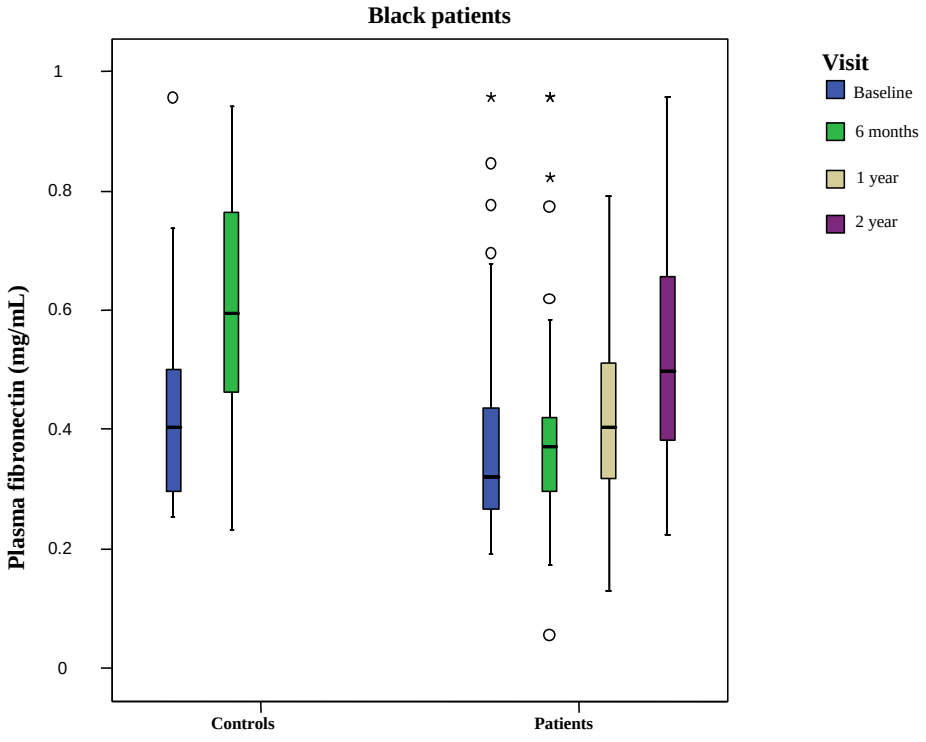

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Figure 31. Box plot of plasma fibronectin in Black control and patient groups. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.8.2 Plasma FN in Indian patients

At baseline and 6-months follow-up, median plasma FN in patients was significantly lower ($p \leq 0.05$) than in the control population (baseline: patients 0.29 mg/mL *vs* control: 0.35 mg/mL; 6 months: patients 0.31 mg/mL *vs* control: 0.35 mg/mL). At baseline, 6-months and 1-year follow-up, median plasma FN values were within the normal range of 0.20 to 0.40 mg/mL in patients (Figure 32). There was a significant increase ($p \leq 0.001$) in the median plasma FN values in patients at 2-year follow-up compared to that at baseline (baseline: 0.28 mg/mL vs 2-year: 0.51 mg/mL).

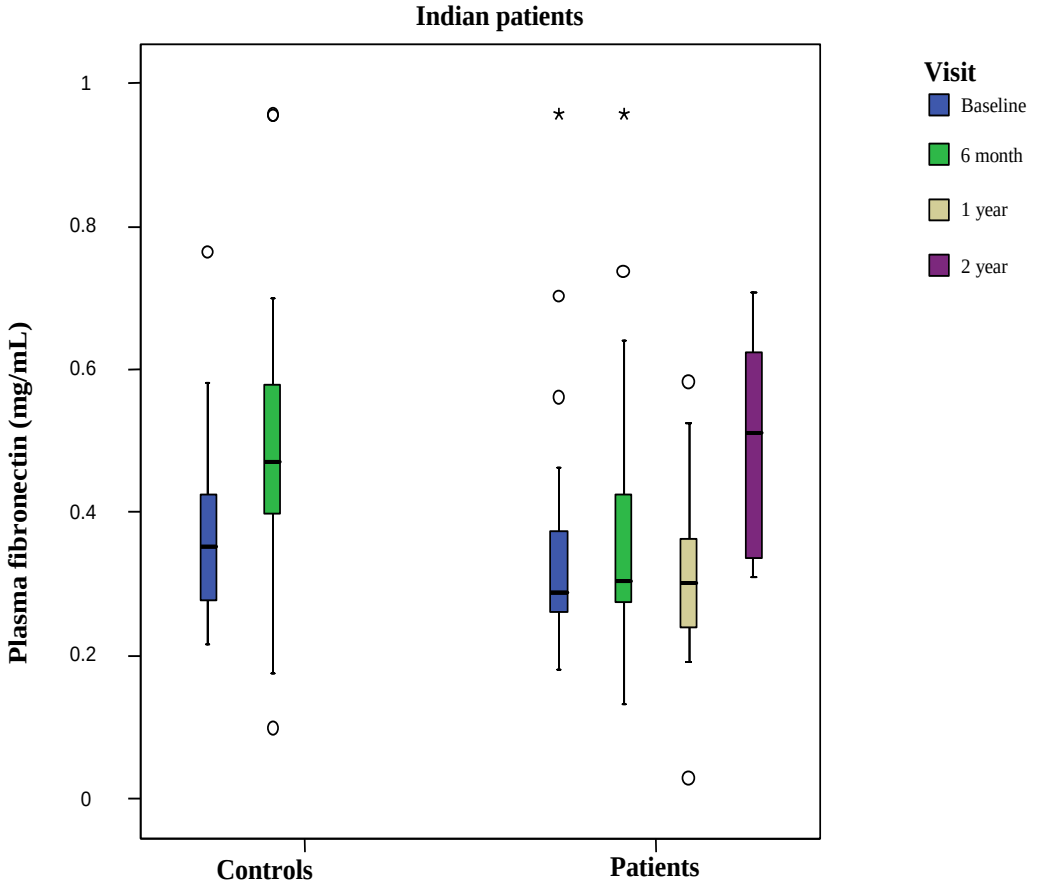


Figure 32. Box plot of plasma fibronectin in Indian control and patient groups. Bars show median value. Box shows the interquartile range (IQR). Whisker shows 1.5 times the IQR. Outliers are values which lie 1.5 times above or below the IQR.

3.2.9 Correlation analysis of isotope GFR and estimates of GFR in patients with diabetes at each visit.

Bi-variate correlations were performed in SPSS, using Spearman correlation co-efficients, comparing estimated GFR values with isotope GFR. A correlation coefficient of 0.75 and above was considered to indicate a strong relationship between variables. A significant correlation was indicated by $p \leq 0.05$. A significant correlation did not indicate a strong correlation, significance being more a function of sample numbers of interrelationship between variables.

The results of the correlations at each visit are shown in Table 8 to 11. Correlations had to be performed independently at each time point. The results indicated that at 1-year and 2-year follow-up, significant correlations ($p \leq 0.05$) existed between isotope GFR and estimates of GFR (CG, 4-variable MDRD and revised 4-variable MDRD), and among the various estimates of GFR themselves, but that these correlations were weak (correlation co-efficient < 0.75) and not consistent across visits. It is for this reason the ethnic groups were combined and analysis performed to determine if a stronger and more consistent correlation across visits was evident. Estimated GFR (eGFR) using the 4-variable MDRD equation showed a correlation of 1.00 ($p \leq 0.001$) with eGFR calculated using the revised 4-variable MDRD equation, which is not surprising considering that the revised equation differs from the original equation only in an ethnicity factor. Overall, correlation analysis indicated that isotope GFR and estimated GFR correlated only weakly with each other.

Table 8. Correlation (as indicated by correlation co-efficient) among estimated glomerular filtration rate (eGFR) using the Cockcroft-Gault (CG), 4-variable Modification of Diet in Renal Disease (4-v MDRD), revised 4-variable MDRD (revised 4-v MDRD) equations, and isotope GFR at baseline.

	Isotope GFR	CG	4-v MDRD	Revised 4-v MDRD
CG	0.558*	-	0.714**	0.714**
4-v MDRD	0.269	0.714**	-	1.000**
Revised 4-v MDRD	0.269	0.714**	1.000**	-

* * $p \leq 0.01$
* $p \leq 0.05$

Table 9. Correlation (as indicated by correlation co-efficient) among estimated glomerular filtration rate (eGFR) using the Cockcroft-Gault (CG), 4-variable Modification of Diet in Renal Disease (4-v MDRD), revised 4-variable MDRD (revised 4-v MDRD) equations, and isotope GFR at 6-month follow-up.

	Isotope GFR	CG	4-v MDRD	Revised 4-v MDRD
CG	0.300		0.576*	0.576*
4-v MDRD	-0.400	0.576**		1.000**
Revised 4-v MDRD	-0.400	0.576**	1.000**	

** $p \leq 0.01$
* $p < 0.05$

Table 10. Correlation (as indicated by correlation co-efficient) among estimated glomerular filtration rate (eGFR) using the Cockcroft-Gault (CG), 4-variable Modification of Diet in Renal Disease (4-v MDRD), revised 4-variable MDRD (revised 4-v MDRD) equations, and isotope GFR at 1-year follow-up.

	Isotope GFR	CG	4-v MDRD	Revised 4-v MDRD
CG	0.455*	-	0.443**	0.443**
4-vMDRD	0.491*	0.443**	-	1.00*
Revised 4-v MDRD	0.491*	0.443**	1.00**	-

** $p \leq 0.01$
* $p \leq 0.05$

Table 11. Correlation (as indicated by correlation co-efficient) among estimated glomerular filtration rate (eGFR) using the Cockcroft-Gault (CG), 4-variable Modification of Diet in Renal Disease (4-v MDRD), revised 4-variable MDRD (revised 4-v MDRD) equations, and isotope GFR at 2-year follow-up.

	Isotope GFR	CG	4-vMDRD	Revised 4-v MDRD
CG	0.623**	-	0.620**	0.620**
4-v MDRD	0.553**	0.620**	-	1.00**
Revised 4-v MDRD	0.553**	0.620**	1.00**	-

** $p \leq 0.01$
* $p \leq 0.05$

3.2.10 Correlation analysis of measures of renal function and indicators of renal function

Correlation studies comparing correlations among the measures of renal function (isotope GFR, CG, 4-variable MDRD and revised 4-variable MDRD) and indicators of renal function (MA, serum PGA, urinary type IV collagen and plasma FN), showed that almost all correlations were weak (correlation coefficient < 0.75), although there were instances of significance of correlations ($p \leq 0.05$) (Tables 12 to 15). Any correlation for which the correlation co-efficient was ≥ 0.75 are shown as shaded cells in the tables. The only consistent strong correlations were between proteinuria and MA. However, this is largely attributable to a study weakness which meant that MA (as an indicator of renal function) and proteinuria (a measure of renal function) were not independent, as explained below. There was no consistency in correlations across visits.

In the correlation analyses and subsequent regression analyses, MA was used as an indicator of renal function, while proteinuria was used as a direct measure of renal function. Microalbuminuria and proteinuria assays both detect protein (albumin) in urine and were used to identify those patients with compromised kidney function. The lower limit for the microalbumin:creatinine ratio (microalbuminuria) is 3.4 mg/mmol creatinine and that for Bayer Multistix 10 SG (proteinuria) is 300 mg/mL. The presence of protein detectable by either method was not truly independent of each other. The routine procedure used in the laboratory for proteinuria measurements was to perform dipstick analysis on urine specimens as an initial screening. Microalbumin analyses were performed if proteinuria was absent. Since the outcome of the proteinuria test determined whether testing for microalbumin was performed, the results of the two tests were not truly independent. It was not initially intended to use proteinuria as a measure of renal function. Only GFR measurement and estimates were intended for this purpose. However, since few patients showed GFR values indicative of overt renal pathology (< 90 mL/min/1.73 m^2), proteinuria was added as an additional measure of renal function in the correlation and regression analyses. This use of proteinuria measurements in a manner not initially included in the study design gave rise to the study weakness introduced by using both microalbumin and proteinuria.

Table 12. Correlation (as indicated by correlation co-efficient) among measures of renal function and indicators of renal function in diabetic patients at baseline.

	Microalbumin	Fibronectin	Collagen IV	Glycated albumin
CG	-0.275	-0.057	0.2180	0.384**
4-v MDRD	-0.220	0.048	0.223	0.290
Revised 4-v MDRD	-0.220	-0.048	0.223	0.290
Isotope GFR	-0.375	-0.534*	-0.316	0.416
Proteinuria	0.847**	0.000	0.866	0.000

****** $p < 0.01$
* $p < 0.05$

Table 13. Correlation (as indicated by correlation co-efficient) among measures of renal function and indicators of renal function in diabetic patients at 6-month follow-up.

	Microalbumin	Fibronectin	Collagen IV	Glycated albumin
CG	-0.352	-0.054	0.606**	0.270
4-variable MDRD	-0.273	-0.158	0.464**	0.312
Revised 4-v MDRD	-0.273	-0.158	0.464**	0.312
Isotope GFR	0.600	-0.300	0.300	-0.300
Proteinuria	0.827**	0.775	-0.258	-0.258

****** $p < 0.01$

Table 14. Correlation (as indicated by correlation co-efficient) among measures of renal function and indicators of renal function in diabetic patients at 1-year follow-up.

	Microalbumin	Fibronectin	Collagen IV	Glycated albumin
CG	-0.207	-0.120	0.032	0.043
4-vMDRD	-0.012	-0.184	-0.277	0.211
Revised 4-v MDRD	-0.012	-0.184	-0.277	0.211
Isotope GFR	-0.186	0.039	-0.259	0.114
Proteinuria	0.805**	0.128	-0.534	-0.155

****** $p < 0.01$

Table 15. Correlation (as indicated by correlation co-efficient) among measures of renal function and indicators of renal function in diabetic patients at 2-year follow-up.

	Microalbumin	Fibronectin	Collagen IV	Glycated albumin
CG	-0.423*	0.029	-0.198	-0.241
4-v MDRD	-0.126	-0.171	-0.224	-0.133
Revised 4-v MDRD	-0.126	-0.171	-0.224	-0.133
Isotope GFR	-0.156	0.112	-0.096	-0.273
Proteinuria	0.785**	0.054	0.551	0.189

** $p \leq 0.01$
* $p < 0.05$

3.2.11 Regression analysis

Regression analysis using generalised estimating equation (GEE) models was performed to identify predictive relationships among the measures of renal function (isotope GFR, various eGFR and proteinuria) over the patient visits (*i.e.* over time). Each measure in turn was set as the dependent variable and the remaining measures were set as independent variables. Time was included as a factor, allowing for variation over time to be considered. Parameter estimates with $p \leq 0.05$ were considered indicative of a significantly predictive relationship. These are shown as shaded cells in the tables, which follow.

Thereafter, regression analysis using GEE models was performed using the measures of renal function (isotope GFR, eGFR using CG, 4-v MDRD and revised 4-v MDRD formulae, and proteinuria), sequentially, as dependent variables, and the indicators of renal function (microalbumin, PGA, urinary type IV collagen and plasma FN) as independent variables. Other independent variables were years of treatment, age, weight, BMI, creatinine, HbA_{1C}, and blood pressure. Initially, only the identified indicators of renal function and measures of renal function were used for the analysis. When this yielded very few predictive relationships, the remaining variables were added. Time was included as a factor. Independent variables which yielded a parameter estimate with $p \leq 0.05$ were considered to be significant predictors of the dependent variable. For those relationships which were found to be significantly predictive, the slope parameter β provided a description of the nature of the relationship. A positive value of β indicated that increase in the independent variable was related to increase in the dependent variable. A negative value of β indicated an inverse relationship, *i.e.* increase in the independent variable was linked to a decrease in the dependent variable. The confidence interval (CI) associated with β indicated the degree of uncertainty associated with the corresponding relationship. A small CI indicated a relatively smaller uncertainty while a large CI indicated a relatively larger uncertainty.

3.2.11.1 Interrelationships among measures of renal function

For all data together (Table 16), isotope GFR significantly predicted eGFR (CG, 4-v MDRD and revised 4-v MDRD). Estimated GFR (CG) significantly predicted isotope GFR and eGFR (4-v MDRD and revised 4-v MDRD). Proteinuria significantly predicted isotope GFR. For all the relationships identified as significantly predictive (shaded table cells), the corresponding values for the slope parameter β and the associated confidence interval (CI) are shown in Table 17. A positive β score indicated a direct relationship *e.g.* increasing isotope GFR with increasing eGFR) (Table 17). For proteinuria the negative β score indicated an inverse relationship *i.e.* increasing proteinuria with decreasing isotope GFR). None of the measures of renal function predicted proteinuria.

When patients groups were separated, a clearer picture emerged. For Black patients, isotope GFR predicted CG. The eGFR (CG), significantly predicted isotope GFR, 4-v MDRD and revised 4-v MDRD, but not proteinuria (Table 18). Proteinuria significantly predicted isotope GFR. For all the relationships identified as significantly predictive (shaded table cells), the corresponding values for the slope parameter β and the associated confidence interval (CI) are shown in Table 19. All of the independent variables showed a positive β score, indicating a direct relationship *e.g* increasing eGFR (CG) with increasing isotope GFR). The only exception was the relationship of proteinuria to isotope GFR. None of the renal function measures predicted proteinuria. For Indian patients, isotope GFR significantly predicted eGFR (CG) and proteinuria. The eGFR (CG) significantly predicted isotope GFR, 4-v MDRD and revised 4-v MDRD. Proteinuria, significantly predicted eGFR (4-v MDRD and revised 4-v MDRD), but not eGFR (CG). The CG, was significantly predicted by isotope GFR, 4-v MDRD and revised 4-v MDRD and proteinuria (Table 20). Estimated GFR (CG) strongly predicted all of the measures of renal function. Isotope GFR and eGFR (CG) significantly predicted proteinuria. In Indian patients all of the independent variables showed a positive β score indicating a direct relationship (Table 21). The only exception was the relationship of eGFR (GG) with proteinuria.

Table 16. Significance of parameter estimates for regression models of interrelationships among measures of renal function for total patient population. Significant relationships ($p \leq 0.05$) are highlighted.

	Dependent Variables				
Independent variables	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Isotope GFR mL/min	-	0.010	0.012	0.012	0.409
CG mL/min	0.004	-	0.000	0.000	0.212
4-v MDRD mL/min/1.73 m^2	0.100	0.000	-	-	0.290
Revised 4-v MDRD mL/min/1.73 m^2	0.100	0.000	-	-	0.290
Proteinuria mg/mL	0.029	0.302	0.388	0.950	-

Table 17. Slope parameter β and confidence interval (CI) for interrelationships among measures of renal function for total patient population, which were significant ($p \leq 0.05$) as per Table 16. CI shown in brackets

	Dependent variables				
Independent variables	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Isotope GFR mL/min	-	0.484 (0.1 – 0.9)	0.365 (0.08 –0.6)	0.301 (0.07 – 0.5)	-
CG mL/min	0.197 (0.06 – 0.3)	-	0.400 (0.2 – 0.6)	0.330 (0.2 – 0.5)	-
4-v MDRD mL/min/1.73 m^2	-	0.528 (0.3 – 0.8)	-	-	-
Revised 4-v MDRD mL/min/1.73 m^2	-	0.528 (0.3 – 0.8)	-	-	-
Proteinuria mg/mL	-0.002 (-0.003 – 0.0)	-	-	-	-

Table 18. Significance of parameter estimates for regression models of interrelationships among measures of renal function in Black patients. Significant relationships ($p \leq 0.05$) are highlighted.

	Dependent Variables				
Independent variables	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Isotope GFR mL/min	-	0.003	0.107	0.100	0.342
CG mL/min	0.026	-	0.047	0.000	0.227
4-v MDRD mL/min/1.73 m^2	0.237	0.000	-	-	0.559
Revised 4-v MDRD mL/min/1.73 m^2	0.237	0.000	-	-	0.559
Proteinuria mg/mL	0.013	0.216	0.573	0.089	-

Table 19. Slope parameter β and confidence interval (CI) for interrelationships among measures of renal function for Black patient population, which were significant ($p \leq 0.05$) as per table 18. CI shown in brackets.

	Dependent variables				
Independent variables	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Isotope GFR mL/min	-	0.445 (0.2 – 0.7)	-		-
CG mL/min	0.249 (0.03 – 0.5)	-	0.471 (0.007 – 0.9)	0.389 (0.006 – 0.8)	-
4-v MDRD mL/min/1.73 m^2	-	0.404 (0.2 – 0.6)	-	-	-
Revised 4-v MDRD mL/min/1.73 m^2	-	0.404 (0.2 – 0.6)	-	-	-
Proteinuria mg/mL	-0.002 (-0.003 – 0.0)	-	-	-	-

Table 20. Significance of parameter estimates for regression models of interrelationships among measures of renal function in Indian patients. Significant relationships ($p \leq 0.05$) are highlighted.

	Dependent variables				
Independent variables	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Isotope GFR mL/min	-	0.012	0.099	0.099	0.021
CG mL/min	0.000	-	0.000	0.000	0.004
4-v MDRD mL/min/1.73 m^2	0.191	0.005	-	-	0.069
Revised 4-v MDRD mL/min/1.73 m^2	0.191	0.005	-	-	0.069
Proteinuria mg/mL	0.098	0.122	0.000	0.000	-

Table 21. Slope parameter β and confidence interval (CI) for interrelationships among measures of renal function for Indian patient population, which were significant ($p \leq 0.05$) as per table 20. CI shown in brackets.

	Dependent variables				
Independent variables	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Isotope GFR mL/min	-	0.740 (0.2 – 1.3)	-	-	4.999 (0.8 – 9.2)
CG mL/min	0.207 (0.1 – 0.3)	-	0.393 (0.3 – 0.5)	0.325 (0.2 – 0.4)	-2.088 (-3.5 - -0.7)
4-v MDRD mL/min/1.73 m^2	-	0.828 (0.2 – 1.4)	-	-	-
Revised 4-v MDRD mL/min/1.73 m^2	-	0.828 (0.2 – 1.4)	-	-	-
Proteinuria mg/mL		-	0.028 (0.02 – 0.04)	0.024 (0.01 – 0.03)	-

3.2.11.2 Relationships of indicators of renal function to measures of renal function

When all patient data were analysed, as a group, the proposed indicator of renal function serum PGA did not predict any of the renal function measures. Plasma FN significantly predicted eGFR (4-v MDRD and revised 4-v MDRD). Urinary type IV collagen significantly predicted eGFR (4-v MDRD and revised 4-v MDRD) (Table 22). Microalbuminuria significantly predicted isotope GFR, eGFR (CG, revised 4-v MDRD) and proteinuria. The relationship between MA and proteinuria is not unexpected as explained earlier.

When study populations were separated into Black and Indian patient groups, different pictures emerged per ethnic group (Tables 24 and 26). In Black patients, serum PGA significantly predicted eGFR (4-v MDRD) (Table 24). Plasma FN significantly predicted eGFR (4-v MDRD). Urinary type IV collagen significantly predicted isotope GFR. Microalbumin significantly predicted proteinuria. In Indian patients, neither serum PGA nor plasma FN significantly predicted any of the renal function measures (Table 26). Urinary type IV collagen significantly predicted eGFR (CG). Microalbuminuria significantly predicted eGFR (4-v MDRD and revised 4-v MDRD) and proteinuria.

When the remaining variables were added to the GEE regression models, in the total patient population serum creatinine significantly predicted isotope GFR, eGFR (4-v MDRD and revised MDRD). Pulse significantly predicted eGFR (CG). Systolic blood pressure significantly predicted eGFR(4-v MDRD and revised 4-v MDRD). Age significantly predicted eGFR (CG and 4-v MDRD). Weight significantly predicted isotope GFR, eGFR (CG). Body mass index significantly predicted eGFR (CG, 4-v MDRD and revised 4-v MDRD). Table 23 shows the β score and CI for each of these variables. Positive β score indicates a direct relationship. A negative β score indicates an inverse relationship *i.e* an increase in the independent variable is associated with a decrease in the dependent variable.

In the Black patients, serum creatinine significantly predicted isotope GFR, eGFR (CG, 4-v MDRD and revised 4-v MDRD) (Figure 24). Systolic blood pressure significantly predicted eGFR (CG). Age significantly predicted eGFR (CG, 4-v MDRD and revised 4-v MDRD). Weight significantly predicted eGFR (CG). Body mass index significantly predicted eGFR (CG, 4-v MDRD and revised 4-v MDRD). Table 25 shows the β score and CI for each of these variables.

In Indian patients, serum creatinine significantly predicted isotope GFR, eGFR (CG, 4-v MDRD and revised 4-v MDRD) (Figure 26). Diastolic blood pressure significantly predicted isotope GFR. Fasting plasma glucose significantly predicted eGFR (4-v MDRD and revised 4-v MDRD). Glycated haemoglobin (HbA1c) significantly predicted isotope GFR. Age significantly predicted isotope GFR and eGFR (CG). Years on treatment significantly predicted isotope GFR. Weight significantly predicted isotope GFR and eGFR (CG). Body mass index significantly predicted eGFR (4-v MDRD and revised 4-v MDRD). Table 27 shows the β score and the CI for each of these variables, indicating the measure of uncertainty associated with the independent variables. In Indian patients more independent variables significantly predict isotope GFR, which is lacking in Black patients. Overall, more predictive relationships were identified for Indian patients than for Black patients. There was little correspondence between patient groups with respect to the relationships identified as being significantly predictive.

Table 22. Significance of parameter estimates for regression models of relationships of functional measures and indicators of renal function (independent variables) to measures of renal function (dependent variables) in total patient population. Significant relationships ($p \leq 0.05$) are highlighted.

Independent variable	Dependent variable				
	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Serum percentage glycated albumin (PGA) (%)	0.477	0.140	0.901	0.360	0.122
Plasma Fibronectin (mg/mL)	0.170	0.170	0.020	0.023	0.853
Urinary type IV collagen (µg/mL)	0.303	0.318	0.041	0.045	0.089
Microalbuminuria (mg/mmoL creatinine)	0.014	0.001	0.067	0.013	0.018
Serum creatinine (µmol/L)	0.000	0.000	0.000	0.000	0.339
Fasting plasma glucose (FPG) (mmol/L)	0.675	0.863	0.200	0.200	0.190
Haemoglobin A_{1C} (HbA_{1C}) (%)	0.148	0.202	0.326	0.288	0.475
Pulse (bpm)	0.857	0.031	0.060	0.060	0.317
Blood pressure supine systolic (mmHg)	0.899	0.182	0.264	0.948	0.365
Blood pressure supine diastolic (mmHg)	0.591	0.645	0.640	0.851	0.163
Age (years)	0.130	0.000	0.002	0.389	0.523
Years on treatment (years)	0.518	0.979	0.564	0.833	0.863
Weight (kg)	0.044	0.000	0.824	0.824	0.381
Body mass index (BMI) (Kg/m^2)	0.427	0.018	0.000	0.000	0.981

Table 23. Slope parameter β and confidence interval (CI) for interrelationships among indicators of renal function (independent variables) to measures of renal function (dependent variables) for total patient population which were significant ($p \leq 0.05$) as per table 22. CI are shown in brackets.

	Dependent variable				
Independent variable	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Serum percentage glycated albumin (PGA) (%)	-	-	-	-	-
Plasma Fibronectin (mg/mL)	-	-	-0.449 (-0.8 - -0.07)	-0.349 (-0.7 - -0.05)	-
Urinary type IV collagen (μg/mL)	-	-	-181.717 (-356 - -7.1)	-187.033 (-370 - -4.5)	-
Microalbuminuria (mg/mmoL creatinine)	-0.337 (-0.6 - -0.07)	0.2226 (-1.2 - -0.3)	-	-0.608 (-1.1 - -0.1)	0.078 (0.2 – 1.6)
Serum creatinine (μmol/L)	-1.396 (-1.7 - -1.1)	-1.396 (-1.7 - -1.1)	-1.643 (-1.9 - -1.4)	-1.240 (-1.5 - -1.0)	-
Fasting plasma glucose (FPG) (mmol/L)	-	-	-	-	-
Haemoglobin A_{1C} (HbA_{1C}) (%)	-	-	-	-	-
Pulse (bpm)	-	0.172 (0.02 – 0.3)	-	-	-
Blood pressure supine systolic (mmHg)	-	-	-	-	-
Blood pressure supine diastolic (mmHg)	-	-	-	-	-
Age (years)	-	-1.134 (-1.6 – 0.7)	-1.253 (-2.0 - -0.5)	-	-
Years on treatment (years)	-	-	-	-	-
Weight (kg)	0.294 (0.008 – 0.6)	1.876 (1.6 – 2.2)	-	-	-
Body mass index (BMI) (Kg/m^2)	-	1.211 (0.3 – 2.2)	-4.275 -5.8 - -2.8	-3.527 (-4.8 - -2.3)	-

Table 24. Significance of parameter estimates for regression models of relationships of functional measures and indicators of renal function (independent variables) to measures of renal function (dependent variables) in Black patients. Significant relationships ($p \leq 0.05$) are highlighted.

Independent variable	Dependent variable				
	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Serum percentage glycated albumin (PGA) (%)	0.172	0.140	0.002	0.103	0.138
Plasma Fibronectin (mg/mL)	0.965	0.170	0.042	0.127	0.700
Urinary type IV collagen (µg/mL)	0.050	0.318	0.888	0.144	0.075
Microalbuminuria (mg/mmoL creatinine)	0.805	0.588	0.059	0.777	0.021
Serum creatinine (µmol/L)	0.000	0.000	0.000	0.000	0.323
Fasting plasma glucose (FPG) (mmol/L)	0.500	0.830	0.576	0.576	0.115
Haemoglobin A_{1C} (HbA_{1C}) (%)	0.487	0.060	0.645	0.645	0.388
Pulse (bpm)	0.631	0.379	0.538	0.538	0.132
Blood pressure supine systolic (mmHg)	0.513	0.006	0.212	0.212	0.127
Blood pressure supine diastolic (mmHg)	0.555	0.299	0.214	0.214	0.125
Age (years)	0.229	0.000	0.027	0.027	0.484
Years on treatment (years)	0.316	0.368	0.310	0.310	0.512
Weight (kg)	0.422	0.000	0.982	0.982	0.213
Body mass index (BMI) (Kg/m^2)	0.803	0.000	0.001	0.001	0.749

Table 25. Slope parameter β and confidence interval (CI) for interrelationships among indicators of renal function (independent variables) to measures of renal function (dependent variables) for Black patient population which were significant ($p \leq 0.05$) as per table 24. CI are shown in brackets.

	Dependent variable				
Independent variable	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Serum percentage glycated albumin (PGA) (%)	-	-	3.711 (1.3 – 6.1)	-	-
Plasma Fibronectin (mg/mL)	-	-	-0.409 (-0.8 - -0.02)	-	-
Urinary type IV collagen (µg/mL)	-192.5 (-385 - -0.1)	-	-	-	-
Microalbuminuria (mg/mmoL creatinine)	-	-	-	-	1.058 (0.161 – 2.0
Serum creatinine (µmol/L)	-.667 (-1.0 - -0.3)	-1.446 (-1.7 - -1.2)	-1.745 (-2.1 - -1.4)	-1.335 (-1.7 – 1.0)	-
Fasting plasma glucose (FPG) (mmol/L)	-	-	-	-	-
Haemoglobin A_{1C} (HbA_{1C}) (%)	-	-	-	-	-
Pulse (bpm)	-	-	-	-	-
Blood pressure supine systolic (mmHg)	-	0.544 (0.2 – 0.9	-	-	-
Blood pressure supine diastolic (mmHg)	-	-	-	-	-
Age (years)	-	-1.324 (-1.7 - -0.9)	-1.605 (-2.6 - -0.5)	-1.207 (-2.3 - -0.1)	-
Years on treatment (years)	-	-	-	-	-
Weight (kg)	-	2.00 (1.7 – 2.2)	-	-	-
Body mass index (BMI) (Kg/m^2)	-	2.2 (1.2 – 3.2)	-5.320 (-8.3 - -2.3)	-4.389 (-6.9 - -1.9)	-

Table 26. Significance of parameter estimates for regression models of relationships of functional measures and indicators of renal function (independent variables) to measures of renal function (dependent variables) in Indian patients. Significant relationships ($p \leq 0.05$) are highlighted.

Independent variable	Dependent variable				
	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Serum percentage glycated albumin (PGA) (%)	0.277	0.046	0.052	0.078	0.503
Plasma Fibronectin (mg/mL)	0.290	0.977	0.858	0.519	0.131
Urinary type IV collagen (µg/mL)	0.767	0.042	0.471	0.622	0.076
Microalbuminuria (mg/mmoL creatinine)	0.644	0.369	0.035	0.029	0.012
Serum creatinine (µmol/L)	0.008	0.000	0.000	0.000	0.131
Fasting plasma glucose (FPG) (mmol/L)	0.709	0.640	0.019	0.019	0.809
Haemoglobin A_{1C} (HbA_{1C}) (%)	0.000	0.199	0.411	0.408	0.113
Pulse (bpm)	0.201	0.138	0.179	0.179	0.839
Blood pressure supine systolic (mmHg)	0.448	0.392	0.932	0.416	0.366
Blood pressure supine diastolic (mmHg)	0.017	0.487	0.810	0.589	0.763
Age (years)	0.008	0.000	0.389	0.170	0.400
Years on treatment (years)	0.001	0.886	0.412	0.197	0.871
Weight (kg)	0.000	0.000	0.152	0.844	0.438
Body mass index (BMI) (Kg/m^2)	0.173	0.970	0.000	0.000	0.258

Table 27. Slope parameter β and confidence interval (CI) for interrelationships among indicators of renal function (independent variables) to measures of renal function (dependent variables) for Indian patient population which were significant ($p \leq 0.05$) as per table 26. CI are shown in brackets.

	Dependent variable				
Independent variable	Isotope GFR mL/min	CG mL/min	4-v MDRD mL/min/1.73 m^2	Revised 4-v MDRD mL/min/1.73 m^2	Proteinuria mg/mL
Serum percentage glycated albumin (PGA) (%)	-	3.146 (0.05 – 6.2)	-	-	-
Plasma Fibronectin (mg/mL)	-	-	-	-	-
Urinary type IV collagen (µg/mL)	-	302.161 (10.7 – 594)	-	-	-
Microalbuminuria (mg/mmoL creatinine)	-	-	0.135 0.01 – 0.26	0.118 (0.01 – 0.2)	0.224 (0.05 – 0.3)
Serum creatinine (µmol/L)	-0.606 (-1.1 - -1.17)	-1.212 (-1.8 - -0.6)	-1.487 (-1.7 - -1.2)	-1.078 (-1.3 - -0.8)	-
Fasting plasma glucose (FPG) (mmol/L)	-	-	-1.084 (-2.0 - -0.2)	-0.895 (-1.6 - -0.1)	-
Haemoglobin A_{1C} (HbA_{1C}) (%)	7.749 (4.4 – 11)	-	-	-	-
Pulse (bpm)	-	-	-	-	-
Blood pressure supine systolic (mmHg)	-	-	-	-	-
Blood pressure supine diastolic (mmHg)	1.089 0.196 – 2.0	-	-	-	-
Age (years)	0.988 (0.257 – 1.7)	-1.397 (-2.1 - -0.7)	-	-	-
Years on treatment (years)	-1.212 (-1.9 – 0.5)		-	-	-
Weight (kg)	0.551 (0.3 – 0.8)	1.713 (1.4 – 2.1)	-	-	-
Body mass index (BMI) (Kg/m^2)	-	-	-4.519 (-5.6 - -3.4)	-3.729 (-4.6 - -2.8)	-

CHAPTER 4

DISCUSSION

4.1 SUMMARY OF RESULTS OF PATIENT MONITORING

The following section presents a summary of the findings presented in the previous chapter to provide the context for the discussion which follows.

Hyperglycaemia as indicated by FPG > 7 mmol/L (reference range 3.1 to 6.4 mmol/L) persisted in the total patient population for the duration of the study (Figure 6 and 8). Raised HbA1c values, *i.e.* > 7%, (reference range 4.8 to 6.0%) across visits indicated poor glycaemic control in the preceding four months (Figure 9 and 11). Fasting plasma glucose and HbA_{1c} were measured only in the patient population due to ethical, cost and logistical reasons and thus comparisons between patient and control populations were not possible. In the control population, random plasma glucose measurements confirmed that hyperglycaemia was not present in this population (Table 6 and 7). Serum creatinine values were lower in the patient population than in control population (Figure 21 and 22). The control population was engaged in physical activity at the time of each sampling (controls sampled from healthy manual labourers in a factory). Since serum creatinine varies between the sexes and is influenced by exercise and higher protein diets (Slentz *et al.* 2004; Baxmann *et al.* 2008), this may account for the increase in serum creatinine relative to the patient population who were not engaged in physical exercise (Kallner *et al.* 2008). In this study, there was no assessment of protein intake or the level of physical activity and its hypothesized that due to the intense work performed by the control population this may have been responsible for raising the level of serum creatinine. Also individuals with moderate/intense physical activity are known to present with lower estimated creatinine clearance (CG) and MDRD than sedentary individuals (Baxmann *et al.* 2008). In this study, a similar trend was noted (Figures 15 to 20), confirming the influence of physical activity in the control population.

The level of MA in the Black patient population differed significantly from the control population (Table 6). In Black patients at baseline and visit 2, 40.7%,(22/54) and 28%, 15/54, respectively were microalbuminuric (> 3.5 mg/mmoL Cr), the remainder of the patients were overtly proteinuric (baseline 41% (14/34); visit 2, 46% (25/54) were overtly proteinuric (> 300 mg/mL). The impact of a relatively small number of control subjects with high MA may be responsible for the difference (baseline 25%, 11/45; visit 2, 31%, 5/16). In Indian patients the level of MA did not differ significantly from the control population (Table 7). In the Indian patients at baseline and visit 2, 21% (6/29) and 28%(8/29), respectively were microalbuminuric (> 3.5 mg/mmoL Cr).
The remainder of the patients were overtly proteinuric (baseline: 31% (9/29); visit 2, 41% (12/29).
A significant proportion of normoglycaemic individuals without known renal pathology show MA. This phenomenon does not mean that MA is an unreliable indicator, rather that MA and renal pathology are dynamic processes. Changes in MA over time are more informative to the clinician than an isolated measure.

In both Black and Indian patients at baseline, the level of PGA was higher in the patient group than in the control population (Figure 27 and 28). There was a trend of decreasing values across visits. At 2-year follow-up the level of PGA was comparable to that in the control population. Urinary type IV collagen was higher in the patient population than in the controls for both baseline and 6-month follow-up (Figure 29 and 30). There was a trend of increasing urinary type IV collagen from baseline to 1-year follow-up. At endpoint the median urinary type IV collagen decreased to values comparable to that at baseline. In Black patients, median plasma FN was lower in the patient population compared to the controls (Figure 31). Plasma FN showed a trend of increasing with time. At 2-year follow-up the median values in the Black patient population were higher than the reference range (0.20 to 0.40 mg/mL). In Indian patients, the median plasma FN at baseline, 6-month and 1-year follow up were in the reference range provided by the supplier (0.20 to 0.40 mg/mL) and comparable to the controls (Figure 32). At 2-year follow-up the median values were higher than the reference range (0.20 to 0.40 mg/mL).

Kidney function, as measured by isotope GFR, was assessed a minimum of once a year in the patient population only. The median isotope GFR across visits remained fairly stable (Figures 13 and 14). The normal rate of decline in isotope GFR in patients with diabetes is 1-2/mL/min/year (Hovind *et al.* 2001; MacIsaacs *et al.* 2006). There is an inverse relationship between serum creatinine and GFR. A significant change in serum creatinine is needed to cause a change in GFR (Jones & Lim 2003; Dalton 2010).

In this study, the isotopic GFR remained fairly stable for the duration of the study period. These results indicate that renal function in the patient population was maintained despite MA and proteinuria. Estimated GFR using the 4-variable MDRD, revised 4-variable MDRD and CG equations differed significantly in the patient population than in the controls (Figures 15 to 20). All three equations showed a similar trend of declining eGFR across visits. In both patient populations (Black and Indian), proteinuria persisted for the duration of the study (Figure 24 and 26).

Results for the indicators of renal function (MA, serum PGA, urinary type IV collagen and plasma FN) and measured of renal function (isotope GFR, eGFR, proteinuria) which were the focus of this study, are discussed in greater detail in the following sections.

4.2 INDICATORS OF RENAL FUNCTION

4.2.1 Microalbuminuria (MA)

Microalbuminuria arises from the increased passage of albumin through the glomerular filtration barrier. Microalbuminuria often precedes overt DN in both type 1 and type 2 diabetes (Adler *et al.* 2003; Perkins *et al.* 2007). It is a classical indicator of incipient DN and is a widely used marker of compromised kidney function in both type 1 and type 2 diabetes (Nayak & Roberts 2006; Muttar *et al.* 2010). Microalbuminuria is also a risk factor for DN.

It is however, not specific to DN, as it can be detected in patients with sepsis, other inflammatory conditions, rheumatoid arthritis and in non-diabetic populations (Hillege *et al.* 2001; Hanefoldt *et al.* 2008). It is prevalent in a small percentage of the general population (Atkins *et al.* 2004; Konta *et al.* 2006). In a study of patients that were hypertensive, MA co-existed in diabetic patients and reducing blood pressure reduced MA (Hovind *et al* 2001; Ficociello *et al.* 2007). Furthermore, not all patients with MA progress to overt nephropathy (Anavekar *et al.* 2004; de Zeeuw *et al.* 2004; King *et al.* 2005). Microalbuminuria has also been reported to be a powerful independent risk factor for cardiovascular disease (Mogenson 2003). The use of antihypertensive medication has been shown to reduce MA (Perkins *et al.* 2003; Araki *et al.* 2005). Furthermore, studies have shown that MA regresses far more than progressing to proteinuria (Perkins *et al.* 2003, Hovind *et al.* 2004; Lutale 2007). For these reasons markers/indicators of compromised renal function other than MA were investigated.

Lutale *et al.* (2007) reported a 12% prevalence of MA in patients with type 1 diabetes. Unnikrishnan *et al.* (2007) showed a 2.2 to 26.9% prevalence of MA in Asian Indian patients with overt nephropathy and MA. In this study, 20% (3/15) of Indian patients and 24% (4/17) Black patients had MA at baseline. At 2-year follow-up, 35% (6/17) Indians and 27% (4/15) Blacks had persistent MA. In Black patients that were proteinuric at baseline, 67% regressed to MA at 2-year follow-up. In Indian patients 67% of proteinuric patients regressed to MA at 2-year follow-up. Atkins *et al.* (2004) showed that 6 % of the adult population had MA with or without hypertension. Perkins *et al.* (2007) reported a 34 % prevalence of MA in control subjects. In this study, the level of MA in the control population was 29% (8/28) for Indian patients and 24% (11/45) for Black patients at baseline, slightly lower than that reported by Perkins *et al.* (2007).

In the total patient population, MA did not correlate with isoptope GFR and eGFR (CG, 4-v MDRD and revised 4-v MDRD) except with eGFR (CG) at 2-year follow-up (Table 15). There was a strong and consistent correlation across visits with proteinuria (Table 12 to 15). Generalised estimating equations (GEE) regression models were used to examine the effect of variation over time with all the indictors of renal function (MA, serum PGA, urinary type IV collagen and plasma FN) simultaneously specified as independent variables, and also including other metabolic measures (FPG and HbA_{1c}) in some analysis.

Analysis using GEE regression models showed that various renal measures were predicted by MA (isotope GFR, revised 4-v MDRD and proteinuria) in the total patient population (Table 22). In the Black population, MA significantly predicted proteinuria (Table 24). In the Indian population, MA significantly predicted by eGFR (4-v MDRD and revised 4-v MDRD) and proteinuria (Table 26). These results suggest that MA significantly predicts proteinuria across populations. However, it must be borne in mind that MA and proteinuria are not independent of each other. In the clinical setting, the use of MA to correctly identify patients with compromised renal function seems appropriate.

The present study shows that microalbuminuria frequently regresses in subjects with type 1 diabetes and that significant proportions of normoglycaemic individuals without known renal pathology show MA. This phenomenon does not mean that MA is an unreliable indicator, rather that MA and renal pathology are dynamic processes. Changes in MA over time are more informative to the clinician than an isolated measure. Also, the patients in this study were treated with anti-hypertensive treatment and this together with lowering of systolic blood pressure (< 129 mmHg) may have contributed to the higher rate of remission.

In this study the hypothesis of anti-hypertensive medication and remission of MA was not tested as it was not part of the study objectives. Future studies are needed to adequately address the effect of anti-hypertensive medication and its role in remission of MA.

4.2.2 Glycated albumin (GA)

To date, there is no established reference range for PGA. In this study, the normal reference range (minimum – maximum) was established using PGA from the control groups (Black 1.15% – 6.69%; Indian 1.54% – 10.56%). For both population groups the level of PGA was higher in diabetics (Black 1.65% - 13.08%; Indian 2.74% - 15.25%) than in the control population, as would be expected. Each laboratory should determine normal values based on the characteristics of the population being studied (Exocell, USA, PA). Further studies are needed to determine the reference range for PGA and at which stage of CKD this marker can be useful as a measure of short-term glycaemia or as an indicator of renal pathology.

Serum glycated albumin (GA), which has a shorter half-life (15 to 20 days) than HbA_{1c} (120 days), has been proposed to be a better marker of short term glycaemic control in diabetic patients (Paroni *et al.* 2007; Yoshiuchi *et al.* 2008; Nagayama *et al.* 2009). Glycated haemoglobin (HbA_{1C}) is used globally as a marker of long term glycaemic control (2 to 3 months) (Rohlfing *et al.* 2002; Kirk *et al.* 2006; Kilpatrick 2008). Glycation of circulating proteins, including haemoglobin and albumin, takes place during the life span of these proteins. Several studies have shown that GA is a better marker of glycaemic control than HbA_{1c} in patients with diabetes and in those undergoing haemodialysis (Kouzuma *et al.* 2004; Kalantar-Zadeh *et al.* 2007; Inaba *et al.* 2007; Peacock *et al.* 2008). Glycated albumin is unaffected by changes in red cell survival (Kosecki *et al.* 2005) or albumin concentration (Kouzuma 2004; Inaba *et al.* 2007).
Okada *et al.* (2007) and Fukuoka *et al.* (2008) have shown that in diabetic patients with ESRD, measuring GA identifies those patients at increased risk of mortality and cardiovascular disease.

Glycated albumin has also been linked to pathogenesis of diabetic vascular complications (Hattori *et al.* 2002; Amore *et al.* 2004; Cohen *et al.* 2006; Li and Wang 2010). In animal models, glycated albumin was shown to be preferentially transported across the glomerular filtration barrier (Londono & Bendayan 2001; Londono & Bendayan 2005). Research has shown that albumin modified by Amadori glucose adducts induces changes in the normal GBM which has been associated with DN and hyperglycaemia induced changes. Glycated albumin has been shown to stimulate expression of type IV collagen and FN (Cohen *et al.* 2005; Cohen *et al.* 2006).
Studies have shown that decreasing the level of glycated albumin resulted in attenuation of glomerular filtration rate and dysregulation of collagen IV production (Cohen *et al.* 2007).

Percentage glycated albumin (PGA), calculated as percentage of GA relative to total albumin, has been reported to be a better indicator of glycaemia than HbA_{1c}). In this study, the level of PGA in patients with type 1 diabetes at baseline was significantly higher than in non-diabetic control subjects ($p \leq 0.05$). These results correspond with findings of other studies in patients with type 1 diabetes and varying stages of CKD (Yoshiuchi *et al.* 2008; Freedman *et al.* 2010). There was a trend of declining PGA values across visits for both Black and Indian patients (Figure 27 and 28). This contrasted with FPG (Figure 9 and 11) and HbA1c (Figure 10 and 12) which were elevated in patients at baseline and remained approximately constant throughout the study.

All of the patients were treated with Metformin (glucophage)which possesses antioxidant properties and decreases the production of ROS. Glycated proteins especially albumin, which is present abundantly in human plasma, has been shown to contribute to vascular complication of diabetes (Schram *et al.* 2005). Faure *et al.* (2008) showed that MET protects albumin from the effects of glycation and ROS by protecting the thiol groups independent of hyperglcaemia. In this study it was found that PGA levels decrease with time, independent of the level of glycaemia. However long lived proteins e.g. HbA_{1c} exposed to raised glucose levels still show the effect of glycation.

Percentage GA did not show consistent correlation with any of the measures of renal function across visits (Table 12 to 14). To investigate the predictive ability of PGA towards renal function measures over time, GEE regression models specifying time as a factor were used. In the total patient population, PGA did not significantly predict any of the measures of renal function (Table 22). When the total patient population was separated into Black and Indian patients, a different picture emerged. In Black patients, PGA significantly predicted eGFR (4-v MDRD) ($p = 0.002$, $\beta = 3.7$, CI 1.3 to 6.1) (Table 24 and 25). The positive slope parameter (β) indicates that increasing PGA values were accompanied by an increase in eGFR.

The associated confidence interval (CI) was relatively narrow, indicating a high degree of confidence in the relationship. In Indian patients, PGA significantly predicted eGFR (CG), ($p = 0.046$, $\beta = 3.1$, CI 0.05 – 6.2) (Table 26 and 27). The associated CI was wide, indicating a high degree of variability, suggesting that in Indian patients, PGA cannot be used as a predictor of renal function. Percentage glycated albumin would be expected to predict GFR if impairment in renal function is directly related to glycation mechanisms, and particularly to the presence of glycated albumin (Cohen *et al.* 2005). Isotope GFR was not significantly predicted by PGA although different estimates of eGFR were (rev-MDRD in Blacks, CG in Indians). It is possible the eGFR is more sensitive to early glycation-associated renal impairment than isotope GFR. However, the evidence from this study is not strong enough to assert this with confidence.

4.2.3 Urinary type IV collagen

The development of DN is accompanied by changes in the ECM and specifically the GBM. Research has indicated that type IV collagen, a major component of the GBM and ECM is a better marker of DN than MA (Tan *et al.* 2002; Tashiro *et al.* 2004). As DN progresses, the level of urinary type IV collagen increases, and has therefore been recommended as an indicator of disease onset and progression (Katavetin *et al.* 2006; Whiteside *et al.* 2009). It has been postulated that mesangial matrix expansion in a diabetic milieu is responsible for decreasing the glomerular filtration surface area with a resultant decline in renal function (GFR) in patients with incipient DN (Pozzi *et al.* 2009).

Cohen *et al.* (2005) showed that glycated albumin is responsible for altering the GBM and stimulating the increased production of type IV collagen in mesangial cells. Studies in animal models have shown that mesangial and glomerular cells exposed to high glucose showed increased production of type IV collagen (Pozzi *et al.* 2008; Ha *et al.* 2009; Whiteside *et al.* 2009).
Yoshioka *et al.* (2004) and Sanna-Cherchi *et al.* (2007) showed that urinary type IV collagen excretion was increased in diabetic patients.

No reference range for type IV collagen was supplied by the manufacturer. The reference range (minimum - maximum) was established using data from the Black and Indian control populations (Black: 0.00473 – 0.09570 µg/mL; Indian: 0.00402 – 0.00935 µg/mL). In this study, urinary type IV collagen was significantly increased in patients with type 1 diabetes compared to the corresponding control population (Black: $p \leq 0.001$; Indian: $p \leq 0.05$) (Table 6 and 7). There was a trend of increasing values from baseline to 1-year follow-up in Black patients (Figure 29).
At 2-year follow-up the median urinary type IV collagen concentration was 0.0111µg/mL compared to 0.0106 µg/mL at baseline (non-significant increase, $p = 0.313$). In Indian patients too, there was a trend of increasing urinary type IV collagen from baseline to 1-year follow-up (Figure 30). The median urinary type IV collagen concentration at 2-year follow-up, was 0.0108 µg/mL compared to 0.0088 µg/mL at baseline (significant increase, $p = 0.013$).

Lowering the concentration of GA has been shown to be associated with reduced levels of urinary type IV collagen (Cohen *et al.* 2005, Cohen *et al.* 2006). In this study PGA values decrease with each visit (Figures 27 and 28). A similar trend with urinary type IV collagen concentration was not found, however, collagen IV values decrease to levels comparable to that at baseline for both Black and Indian patients, despite persistent hyperglycaemia (Figure 29 and 30). These results suggest that urinary type IV collagen is a marker that lags behind in comparison to PGA which is a marker of glycation.

In the total patient population, urinary type IV collagen did not correlate significantly with the markers of renal function (Table 12 to 14). Analysis using GEE regression models showed that in the total patient population urinary type IV collagen significantly predicted eGFR (4-v MDRD and revised 4-v MDRD) (Table 22). The negative β parameter and wide CI (Table 23) indicate that the relationship shows high variability. In Black patients, urinary type IV concentration significantly predicted isotope GFR, but none of the other measures of renal function (Table 24). The negative slope parameter (β = -192.5) indicates the isotope GFR decreases as urinary type IV collagen increased (Table 25). The wide confidence interval (–384.8 to –0.1) on the slope parameter indicated that the relationship showed high variability. In Indian patients, urinary type IV collagen significantly predictive eGFR (CG) (Table 26). The positive slope parameter (β = 302.161)
(Table 27) indicated that eGFR (CG) increased as urinary type IV collagen increased, which is confirmed by Figure 20. The wide confidence interval on the slope parameter (CI 10.7 to 594) shows that this relationship is widely variable. This relationship is counter-intuitive and in contrast to literature reports of increasing type IV collagen with increasing renal pathology (decreasing GFR). Thus results suggest that urinary type IV collagen may predict isotope GFR in Black patients but the relationship between GFR and urinary type IV collagen in Indian patients needs to be further elucidated.

4.2.4 Plasma fibronectin (FN)

Plasma FN is a marker of vascular injury and has been shown to be elevated in diabetic patients and (Kanters *et al.* 2001; Shui *et al.* 2006; Wang *et al.* 2008). Work by Jung *et al.* (2008) showed that hyperglycaemia induces increased fibronectin expression in glomeruli mesangial cells *in vitro*. Excessive glucose has been implicated in mediating pathological changes in the tubular basement membrane which alters the composition and function of the matrix, leading to DN (Lee *et al.* 2003; Feng *et al.* 2005; Wang *et al.* 2008). A large portion of the renal tubulointerstitium comprises epithelium, blood vessels and interstitium. The predominant cell type is proximal renal tubular epithelial cells (PTEC). Yung *et al.* (2006) showed that PTEC cells exposed to high glucose levels exhibited increased production of FN. In human cells, high glucose concentration also stimulated increased production of FN (Okada *et al.* 2005; Mason 2009).

For both Black and Indian patients, median plasma FN was within the normal range supplied by the manufacturer of the assay kit (0.20 to 0.40 mg/mL) for baseline, 6-months and 1–year follow-up. For both Black and Indian patients, median plasma FN increased significantly relative to baseline by 2-year follow-up (Figure 31 and 32). Persistent hyperglycaemia, as indicated by raised FPG (Figure 9 and 11) and elevated HbA1c (Figure 10 and 12) across visits, may be responsible for the increased production of plasma FN.

In this study, in the total patient population, plasma FN showed no consistent correlation with isotope GFR, eGFR (CG, 4-v MDRD and revised MDRD equations) and proteinuria across visits (Table 12 to 14). Generalised estimating equations (GEE) regression models showed that in the total patient population plasma FN significantly predicted eGFR (4-v MDRD and revised 4-v MDRD) (Table 22). The negative β slope parameter and wide CI (Table 23) indicate high variability with plasma FN. In Black patients, increasing plasma FN significantly predicted decreasing eGFR, (4-v MDRD, $p = 0.042$, $\beta = -0.409$, CI –0.8 to –0.02) (Table 25). This is borne out by comparison of Figure 12 with Figure 28. Despite plasma FN significantly predicting eGFR (4-v MDRD), it did not significantly predict isotope GFR or proteinuria. In Indian patients, plasma FN did not significantly predict any of the measures of renal function (Table 26).

These results suggest that plasma FN did not significantly predict measures of renal function and its use as a marker of compromised renal function is not supported in South African Black and Indian patients with type 1 diabetes.

4.3 MEASURES OF RENAL FUNCTION

4.3.1 Isotope GFR and estimated GFR

Isotope measurements of GFR are costly and require the expertise of highly trained personnel. To assess GFR in the study population, in the present study, three estimating equations of GFR were used, *viz.* 4-variable MDRD, revised 4-variable MDRD and CG. These estimated GFR values were compared with isotope GFR values where available. Studies have shown that the 4-variable MDRD (Poge *et al.* 2006) and CG equations (Stevens *et al.* 2006) overestimate GFR and this should be borne in mind when using these equations to assess GFR. Numerous methodological problems impact on the accuracy of these estimates. These include the patient population being studied, the statistical method used for analysis, traceability of the method to a calibration standard and the reference isotope GFR method used (Tidman *et al.* 2008).

Both MDRD and CG formulae have been investigated in healthy subjects and those with CKD, and found to be broadly accurate estimates of GFR (Rule *et al.* 2004; Froissart *et al.* 2005; Poggio *et al.*2005). In the clinical setting the 4-variable MDRD equation is widely used. It has been reported to be more accurate and more helpful in correctly identifying those patients with CKD than the CG equation (Rigalleau *et al.* 2005). The MDRD equation incorporates body weight and is recommended for the monitoring of renal function during treatment that influences kidney performance (Rule *et al.* 2004; Poggio *et al.* 2005; Premaratne *et al.* 2008). Lamb *et al.* (2005) reported that the MDRD equation shows the least bias in assessing eGFR and is applicable for follow-up in patients with CKD. In that study, patients were staged as CKD 3 and 4. The results supported the use of the 4-v MDRD equation to monitor diabetic patients with moderate to advanced kidney disease in clinical practice (Poggio *et al.* 2005).

In the present study, isotope GFR remained fairly constant across visits (Figure 13 and 14), indicating that kidney function was maintained for the duration of this study. In contrast, across both population groups, all three equations for eGFR (4-v MDRD, revised MDRD and CG) showed similar trends of declining renal function (Figures 15 to 20). Despite this apparent difference, the eGFR equations showed statistical correlation with each other and with isotope GFR (Table 8 to 11). The estimated values of GFR were within 30% of the isotope GFR value at all times (Tidman *et al.* 2008). All three equations showed a similar trend of declining eGFR across visits (Figures 15-20). The eGFR formulae are based on age, sex, weight and serum creatinine.
Both the CG and 4-variable MDRD equation overestimated GFR for Black and Indian patients (Table 6 and 7). In comparison, the revised 4-v MDRD equation reported values closer to the isotope GFR and were within 10% of this value (Table 6 and 7). This corresponds with results reported by van Deventer *et al.* (2008). The eGFR values (CG, 4-v MDRD and revised 4-v MDRD) showed good correlation with each other (Table 8 to 11). Using GEE regression models, eGFR (CG) significantly predicted isotope GFR in the total patient population (Table 16). The positive β and narrow CI indicate a high degree of confidence in the relationship. In the Black patient population , the eGFR (CG) significantly predicted isotope GFR (Table 18). The positive β and narrow CI indicate a high degree of confidence in the relationship (Table 19). In Indian patients eGFR (CG) significantly predicted isotope GFR (Table 20). The positive β and narrow CI indicate a high degree of confidence in the relationship (Table 21).

Generalised estimating equations (GEE) regression models showed that for the total patient population, only the CG equation was significantly predicted by 4-v MDRD, revised 4-v MDRD (Table 16). Isotope GFR was significantly predicted by eGFR (CG) and proteinuria. In Black patients, most measures were predicted well by the other measures (Table 18). Thus in Black patients all the estimating equations for GFR behaved broadly similarly. Proteinuria significantly predicted isotope GFR (Table 18). Thus this study indicated that proteinuria can be used to predict isotope GFR in Black patients.

In Indian patients, there were no consistent relationships amongst the measures, in contrast to Black patients. Isotope GFR was significantly predicted by eGFR (CG).Estimated GFR (CG) significantly predicted all of the measures of renal function (Table 20). Furthermore, proteinuria was significantly predicted by isotope GFR and CG, but not 4-v MDRD and revised 4-v MDRD. These results suggest that in a clinical setting, caution needs to be exercised when using the 4-v MDRD and revised 4-v MDRD equation to identify Indian patients at risk of DN, but CG equation appears to be a reliable indicator.

4.3.2 Proteinuria

It has been reported that, in untreated patients with persistent MA, 80% of patients with type 1 diabetes progress to overt DN (ADA 2004). Routinely the screening of patients urine with protein dipstix measurements is used to identify those with compromised renal function and institute treatment which may improve the loss of protein. However, over time the loss of renal function eventually results in ESRD (Perkins *et al.* 2007; Perkins *et al.* 2010). Perkins *et al.* (2010) reported that 35% of patients with CKD stage 3-4 developed proteinuria. In this study a small percentage of patients were overtly proteinuric at 2-year follow-up (Black: 26% (9/34), Indian: 10% (2/20).

In the Black patient population, 55% (5/9) of patients that were proteinuric at baseline regressed to MA (< 3.4 mg/mmoL creatinine) and 22% (2/9) had persistent MA (> 3.4 mg/mmoL creatinine) at 2-year follow-up. In the Indian patient population at 2-year follow-up, 25% (1/4) of patients that were initially proteinuric regressed to MA (< 3.4 mg/mmoL creatinine). In order to assess the role of proteinuria in renal function measures, proteinuria was included in the correlation analyses and GEE regression models. Correlation studies showed that proteinuria correlated with plasma FN and serum PGA only at baseline (Table 11), but this was not consistent across visits (Table 12 to 14). Using GEE regression models, proteinuria was significantly predicted by MA in the total patient population (Table 18) as well as in the Black and Indian patient populations individually (Table 19 and 20).

The traditional view that MA progresses to proteinuria requires review. Studies have shown the presence of MA infers a 20-25% risk of progression to proteinuria within 10 years in patients with CKD stage 3-4 (Ficociello *et al.* 2007; Amin *et al.* 2008). However, biomarkers like MA, are not sufficiently robust as markers for the development of CKD in patients with type 1 diabetes. The presence of MA can remain static or advance to proteinuria, but more frequently regresses to normal levels of albumin excretion (Giorgino *et al.* 2004). Renal function can also decline without overt proteinuria. Therefore better markers are needed that can identify patients at risk of advanced CKD many years before its development are needed (Perkins *et al.* 2010).

The association of MA and proteinuria is not truly independent (as discussed previously) and this may be the reason why MA consistently predicted proteinuria. To fully investigate the relationship of MA and proteinuria in South African Black and Indian patients with type I diabetes, further studies are needed.

4.4 RELEVANCE OF PROPOSED INDICATORS OF RENAL FUNCTION

In summary, for the total patient population, at least some of the different measures of eGFR (CG, 4-v MDRD and revised 4-v MDRD) were significantly predicted by PGA, urinary type IV collagen and plasma FN. There was no consistency across population groups and neither of the direct measures of renal function (isotope GFR and proteinuria) was significantly predicted by the proposed new markers. Across the population groups, MA significantly predicted proteinuria (Table 12 to14), and proteinuria in turn predicted isotope GFR. Since these measures are not truly independent of each other, this association was expected but its potential significance should not be excluded. Thus this study suggests that in the clinical setting, the use of MA as an indicator of compromised renal function remains the most useful tool to identify those South African Black and Indian patients at risk of DN.

4.5 OTHER VARIABLES WHICH PREDICTED RENAL FUNCTION

In this study, several variables associated with diabetes, besides the proposed new markers, were shown to significantly predict measures of kidney function. Table 22 shows a summary of these independent variables for the total patient population and their association with renal function. Serum creatinine significantly predicted measures of renal function, especially isotope GFR, across both populations (Table 24 and 26). Proteinuria was the exception to this. Serum creatinine was a stronger predictor of measures of renal function than the proposed new markers of DN. These results show that in clinical practice, increasing serum creatinine is a good marker for identifying patients with incipient declining renal function.

In Indian patients, systolic BP did not show a role in predicting declining kidney function across all measures of renal function (Table 26). In Indian patients with long duration of type 1 diabetes, a larger number of independent variables was shown to be significantly predictive of isotope GFR than in that in Black patients. Surprisingly, despite the high incidence of hypertension in SA Black patients, as reported by Motala *et al.* (2003), systolic BP did not significantly predict isotope GFR or eGFR (4-v MDRD and revised 4-v MDRD) in the Black patient population. However, systolic BP significantly predicted creatinine clearance (CG) in Black patients.

These results suggest that in Black patients, the association of several factors associated with diabetes contribute to progression of disease more strongly than hypertension in isolation. In the two ethnic populations studied, differences were evident in disease progression, reflected in the different physiological and metabolic variables which significantly predicted measures of renal function. Thus differences in physiology and ethnicity need to be borne in mind when monitored disease progression in Black and Indian patients with type 1 diabetes.

For the total patient population, hyperglycaemia (FPG and HbA_{1c}) did not significantly predict any of the measures of renal function (Table 22). For Black patients only, a similar pattern was observed (Table 24). In Black patients, glycaemic indices (FPG and HbA1c) did not predict renal function. In the Black patient population studied, the duration of diabetes and the age of the patients were lower, compared to that of the Indian patients who were much older and had longer duration of diabetes (Table 6 and 7). This may account for the differences observed. Alternatively secondary metabolic mechanisms as discussed in Chapter 1 (e.g. increased polyol pathway flux, increased hexosamine flux, activation of protein kinase C and reactive oxygen species) may play a more important role than glycaemia itself in renal function decline over time. As discussed in Chapter 1, hyperglycaemia activates many metabolic pathways that initiate ROS generation and the formation of AGEs over time, which alter the structure and function of the kidney. In Indian patients, the longer duration of disease processes as well as glycaemic indices were identified as strong predictors of renal function decline.

These results suggest that glycation processes and AGE formation may be directly involved in the disease process. Future studies are required to prove this hypothesis in Indian and Black patients with long duration of diabetes. This suggests that factors associated with diabetes besides hyperglycaemia itself may be responsible for disease progression in Black patients. Identifying and intervening clinically in these factors would be useful in a clinical setting. However, in Indian patients hyperglycaemia (HbA_{1c}) was independently predictive of measures of renal function, especially isotope GFR. This suggests that hyperglycaemia probably plays a direct role in disease progression in Indian patients. Thus steps to correct hyperglycaemia may prove useful in the clinical setting to limit effects of renal function.

Years on treatment, age and weight, were significantly predictive of renal function measures, especially isotope GFR in Indian patients, more so than in Black patients. Thus in Indian patients, hyperglycaemia and the time-associated effects of hyperglycaemia seem to play a role in declining renal function. The role of these variables in predicting renal decline in both population groups with type 1 diabetes needs to be further investigated.

4.6 CONCLUSIONS

It was hypothesised that glycated serum albumin, plasma fibronectin and urinary type IV collagen, either alone or in combination are better predictors of markers of impaired renal function than microalbuminuria. It was further hypothesised that this relationship differs between South African Black and Indian patient populations.

The results of this study showed that serum GA, plasma FN and urinary type IV collagen were not better predictors of incipient impaired renal function than MA.

In addition the following conclusions can be drawn:

- In this prospective study, hyperglycaemia persisted across visits and median HbA_{1c} values were above the therapeutic range of < 7.0%. Therefore results are representative of Black and Indian patients with elevated blood glucose.

- Proteinuria was higher in Black patients at 32% (8/25) of patients at 2-year follow-up than in Indian patients. In Indian patients proteinuria was found in 10% (2/20) at 2-year follow-up.

- Proteinuria was significantly predictive of isotope GFR and could therefore be considered a reliable marker of DN.

- Isotope GFR is still a useful measure of kidney function in diabetic patients. However, results from this study suggest that estimates of GFR should be used with care where renal function is not yet grossly compromised. The revised 4-variable MRDR equation without ethnicity factor can be used to assess GFR in Black patients. In Indian patients the use of the 4-v MDRD and revised 4-v MDRD equation is not reliable to assess GFR. The CG equation was more reliable.

- Percentage glycated albumin cannot be used as a marker of renal function in either Black or Indian patients.

- Urinary type IV collagen may be a useful marker to identify patients at risk of DN in Black patients with type 1diabetes but not in Indian patients.

- Plasma FN cannot be used as a marker of vascular injury to identify patients at risk of DN.

- Microalbuminuria predicted proteinuria in both populations. However, the interdependence of MA and proteinuria measurements cannot be over-emphasized, since both assays measure albumin in urine.

- The most significant finding of this study was that serum creatinine was still the strongest predictor of renal function of all the investigated variables, more so than the proposed new markers.

4.7 SHORTCOMINGS OF THIS STUDY

- The relatively short duration of this study may not be totally representative of decline in renal function in both population groups over extended time periods (*e.g.* decades).

- Loss of patients to follow-up contributed to a smaller number of patients analysed at end-point, meaning that the sample populations may not have been representative of the populations studied.

- Microalbuminuria and proteinuria assays were not truly independent of each other as both assays measure protein (albumin) in urine and the way these were measured in this study meant measurements were not truly independent of each other.

- More frequent isotope GFR determinations may have added value in identifying patients with declining renal function.

- For the duration of this study, only a small percentage of patients had declining renal function. In most published studies, patients selected had clearly compromised renal function. The results presented here are significant in highlighting that markers of overt DN do not necessarily apply as well to incipient DN. However, results presented here require confirmation before being used in clinical practice for patient management.

4.8 RECOMMENDATIONS

4.8.1 Recommendations for Clinical Practice

- Of the investigated markers of early DN, MA was the most consistently predictive of renal function measures, therefore it is recommended that in clinical practice MA sill be used as such. However, MA needs to be interpreted with caution, as a noticeable number of patients with MA regressed to normal levels, more so than progressed to proteinuria.

- Creatinine emerged as a strong predictor of renal function in Black and Indian populations in which GFR was not yet severely compromised. It is therefore to be considered a strong predictor of early DN.

- The results of this study indicate that there is evidence for different disease processes in early DN in South African Black and Indian patients with type 1 diabetes.

4.8.2 Recommendation for Further Research

- More research is needed into markers of early DN, as opposed to overt DN. This study demonstrated that markers proposed on the basis of studies in patients with overt DN do not necessarily apply in the early stages of compromised renal function.

- The difference in disease processes between Black and Indian patients requires further investigation, in particular the observation that DN in Black patients does not appear to be directly predicted by either fasting plasma glucose or HbA_{1c}.

- The low renal function relative to the high level of MA in Black control subjects needs to be further investigated.

REFERENCES

Abe H, Matsubara T, Iehara N, Nagai N, Nagai K, Takahashi T, Arai H, Kita T and Doi T. (2004). Type IV Collagen is transcriptionally regulated by Smad1 under Advanced Glycation End Product (AGE) Stimulation. *The Journal of Biological Chemistry* 270:14201-14206.

Adler AI, Stevens RJ, Manley SE, Bilous RW, Cull CA and Holman RR. (2003). Development and progression of nephropathy in type 2 diabetes: United Kingdom Prospective Diabetes Study (UKPDS 64). *Kidney International* 63:225-232.

Åkerblom HK, Vaarala O, Hyöty H, Honen J and Knip M. (2002). Environmental factors in the etiology of type 1 diabetes. *American Journal of Medicine and Genetics* 115:18-29.

Alebiosu CO, Kadiri S and Akang E.E.U. (2002). Clinicopathological study of Diabetic Nephropathy based on Renal Biopsy. *Diabetes International* 12:66-69.

Alexandraki K, Piperi C, Kalofoutis C, Singh J, Alaveras A and Kalofoutis A. (2006). The inflammatory process in type 2 diabetes. The role of cytokines. *Annals of New York Academy of Sciences* 1084:89-117.

American Diabetes Association. (2002). Diabetic nephropathy. *Diabetes Care* 25, Suppl 1:S85-S89.

American Diabetes Association. (2004). Tests of glycaemia in Diabetes. *Diabetes Care* 27:S90-S93.

American Diabetes Association. (2005). Diagnosis and Classification of diabetes mellitus. *Diabetes Care.* 28:S37-S42.

American Diabetes Association. (2010). Executive Summary: Standards of Medical Care in Diabetes – 2010. *Diabetes Care* (33-1) Supplement 1. S4-S10.

Amin R, Turner C, van Aken S, Bahu TK, Watts A, Lindsell DR, Dalton RN, and Dunger DB. (2005). The relationship between microalbuminuria and glomerular filtration rate in young type 1 diabetic subjects: The Oxford Regional Prospective Study. *Kidney International* 68:1740-1749.

Amin R, Widmer B, Provost, Schwarze P, Cooper J, Edge J, Marcovecchio L, Neil A, Dalton RN and Dunger DB. (2008). Risk of microalbuminuria and progression to macroalbuminuria in a cohort with childhood onset type 1 diabetes: prospective observational study. *British Medical Journal 336:*697-701.

Amore A, Cirna P, Conti G, Cerutti F, Bagheri N, Emancipator SN and Coppo R. (2004). Amadori-configurated albumin induces nitric oxide-dependent apoptosis of endothelial cells: A possible mechanism of diabetic vasculopathy. *Nephrology Dialysis Transplantation* 19:53-60.

Anavekar NS, McMurray JJ, Velazquez EJ, Scott D, Solomon SD, Kober L, Roleau JL. White HD, Nordlander R, Maggioni A, Dickstein K, Zelenkofske S, Leimberger JD, Califf RM, and Pfeffer MA. (2004). Relation between renal dysfunction and cardiovascular outcomes after myocardial infarction. *New England Journal of Medicine* 351:1285-1295.

Araki S, Haneda M, Sugimoto T, Isono M, Isshiki K, Kashiwagi A and Koya D. (2005). Factors associated with frequent remission of microalbuminuria in patients with type 2 diabetes mellitus. *Diabetes 54:*2983-2987.

Arogundade FA and Barsoum RS. (2008). CKD prevention in sub-Saharan Africa: a call for governmental , nongovernmental and community support. *American Journal of Kidney Disease* 51:515-523.

Ashley EA, Spin JM, Tabibizaar R and Quertermous T. (2007). Frontiers in Nephrology: Genomic Approaches to understanding the Molecular Basis of Atherosclerosis. *Journal of the American Society of Nephrology* 18:2853-2862.

Asnaghi V, Gerhardinger C, Hoehn T, Abeboje A and Lorenzi. (2003). A role for the polyol pathway in the early neuroretinal apoptosis and glial changes induced by diabetes in the rat. *Diabetes* 52 :506-511.

Atkins RC, Polkinghorne KR, Briganti EM, Shaw JE, Zimmet PZ and Chadban SJ. (2004). Prevalence of Albuminuria in Australia: The AusDiab Kidney Study. *Kidney International* 66: S22-S24.

Awad AS, Webb RL, Carey RM, and Siragy HM. (2005). Increased renal production of angiotensin II and thromboxane B2 in conscious diabetic rats. *American Journal of Hypertension* 18:544-548.

Ayodele OE, Alebiosu CO and Salako BL. (2004). Diabetic Nephropathy-A Review of the Natural History, Burden, Risk Factors and Treatment. *Journal of the National Medical Association* 96:1445-1452.

Bangstad HJ, Osterby R, Rudberg S, Hartman A, Bradbrand K and Hanssen KF. (2002). Kidney function and glomerulopathy over 8 years in young patients with type I (insulin-dependent) diabetes mellitus and microalbuminuria. *Diabetologia* 45:253-261.

Baxmann AC, Ahmed MS, Marques NC, Menon VB, Pereir AP, Kirstajn GM and Heilberg IP. (2008). Influence of Muscle mass and Physical activity on Serum and Urinary Creatinine and Serum Cystatin C. *Clinical Journal of the American Society of Nephrology* 3:348-354.

Bostom AG, Kronenberg F and Ritz E. (2002). Predictive performance of renal function equations for patients with chronic kidney disease and normal serum creatinine levels. *Journal of the American Society of Nephrology* 13:2140-2144.

Brownlee M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820.

Bry L, Chen PC and Sacks DB. (2001). Effects of Hemoglobin Variants and Chemically Modified Derivatives on Assays for Glycohemoglobin. *Clinical Chemisty* 47: 153-163.

Bucala R , Vlassara H and Cerami A. (1994). Advanced glycosylation endproducts: Role in diabetic and non-diabetic vascular disease. *Drug Development Research* 32:77.

Burns WC, Twigg SM, Forbes JM, Pete J, Tikellis C, Thalla-Bonke V, Thomas MC, Cooper ME and Kantharidis P. (2006). Connective tissue growth factor plays an important role in advanced glycation end-product induced tubular epithelial-to-mesanchymal transition: implications for diabetic disease. *Journal of the American Society of Nephrology* 17:2484-2494.

Buse MG. (2006). Hexosamines, insulin resistance, and the complications of diabetes: current status. *American Journal of Physiology Endocrinology and Metabolism* 290: E1-E8.

Cade WT. (2008). Diabetes-Related Microvascular and Macrovascular Diseases in Physical Therapy Setting. *Physical Therapy* 88: 1322-1335.

Calvo LC, Cothe P, Fernandez PG, de Alvaro F, Perez CF and RICARHD investigators. (2006). Target organ damage and cardiovascular complications in patients with hypertension and type 2 diabetes in Spain: a cross-sectional study. *Cardiovascular Diabetology* 5:23. doi:10.1186/1475-2840-5-23.

Caramori ML, Fioretto P, and Mauer M. (2000). The need for early predictors of diabetic nephropathy risk: Is albumin excretion rate sufficient? *Diabetes* 49:1399-1408.

Caramorri ML, Fioretto P and Mauer M. (2003). Low glomerular filtration rate in normoalbuminuric type 1 diabetic patients: An indicator of more advanced glomerular lesions. *Diabetes* 52:1036-1040.

Chen S, Kasama Y, Lee JS, Jim B, Martin M and Ziyadeh FN. (2004). Podocyte-derived vascular endothelial growth factor mediates stimulation of α3(IV) collagen production by transforming growth factor-β1 in mouse podocytes. *Diabetes* 53:2939-2949.

Chen S, Lee JS, Iglesias-de la Cruz MC, Wang A, Izquierdo-Lahuerta A, Gandhi NK, Danesh FR, Wolf G and Ziyadeh FN. (2005). Angiotensin II stimulates A3(IV) collagen productions in mouse podocytes via TGF-β and VEGF signalling: implications for diabetic glomerulopathy. *Nephrology Dialysis Transplantation* 20:1320-1328.

Cherian S, Roy S, Pinheiro A and Roy S. (2009). Tight Glycaemic Control Regulates Fibronectin Expression and Basement Membrane Thickening in Retinal and Glomerular Capillaries of Diabetic Rats. *Investigative Ophthalmology and Visual Science 50*:943-949.

Cho J and Mosher DF. (2006). Role of fibronectin assembly in platelet thrombus formation. *Journal of Thrombotic Heamostasis* 4:1461-1469.

Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL Jr, Jones DW, Materson BJ, Oparil S, Wright JT Jr and Roccella EJ. (2003). The Seventh Report of the Joint National Committee on Prevention , Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. *Journal of the American Medical Association* 289:2560-2572.

Christensen PK. (2004). Renal structure and function in type 2 diabetic patients with or without diabetic nephropathy. *Danish Medical Bulletin* - No. 1. 51:82-103.

Chung SSM, Ho ECM, Lam KSL and Chung SK. (2003). Contribution of Polyol Pathway to Diabetes-Induced Oxidative Stress. *Journal of the American Society of Nephrology* 14:S233-S236.

Cirillo M, Anastasio P and De Santo N. (2005). Relationship of gender, age, and body mass index to errors in predicted kidney function. *Nephrology Dialysis Transplantation* 20:1791 – 1798.

Cockcroft DW and Gault MH. (1976). Prediction of creatinine clearance from serum creatinine. *Nephron Journals*16:31-41.

Cohen MP, Chen S, Ziyadeh FN, Shea E, Hud EA, Lautenslager GT and Shearman CQ. (2005). Evidence linking glycated albumin to altered glomerular nephrin and VEGF expression, proteinuria and diabetic nephropathy. *Kidney International* 68:1554-1561.

Cohen MP, Lautenslager GT, Shea E, Wang A, Chen S and Shearman CW. (2007). Inhibiting albumin glycation attenuates dysregulation of VEGFR-1 and collagen IV subchain production and the developemnt of renal insufficiency. *American Journal of Physiology Renal Physiology* 292:F789-F795.

Cohen MP. University City Science Centre, 3508 Market Stret, Philadelpgia, PA 19104. E-mail:drmpcohen@aol.com.

Cohen MP, Lautenslager GT and Shearman CW. (2001). Increased Collagen IV Excretion in Diabetes - A marker of compromised filtration function. *Diabetes Care* 24:914-918.

Cohen MP, Shea E, Chen S and Shearman CW. (2003). Glycated albumin increases oxidative stress , activates NF-κB and extracellular signal regulated kinase (ERK), and stimulates ERK-dependent transforming growth factor-β1 production in macrophage RAW cells. *Journal of Laboratory Clinical Medicine 141*:22-29.

Cohen MP, Ziyadeh FN and Chen S. (2006). Amadori-modified glycated serum proteins and accelerated atherosclerosis in diabetes: Pathogenic and therapeutic implications. *Journal of Laboratory and Clinical Medicine* 147:211-219.

Colagiuri. S. (2006). WHO/IDF Guidelines on the diagnostic criteria for diabetes and impaired glycemic regulation.2006 Diabetes UK, *Diabetic Medicine* 23:200-410.

Coresh J, Eknoyan G and Levey AS. (2002). Estimating the prevalence of low glomerular filtration rate requires attention to the creatinine assay calibration. *Journal of American Society of Nephrology* 13:2811-2112.

Crook M. (2004). Type 2 diabetes mellitus: A disease of the innate immune system? An update. *Diabetes Medicine* 21:203-207.

Cutler RG. (2005). "Oxidative stress profiling-part I. Its potential importance in the optimization of human health". *Annals of the New York Academy of Sciences* 1055:93-135.

Dagher Z, Park YS, Asnaghi V, Hoehn T, Gerhardinger C and Lorenzi M. (2004). Studies on rate and human retinas predict a role for the polyol pathway in human diabetic retinopathy. *Diabetes* 53:2404-2411.

Dalla VM, Masiero A, Roiter AM, Saller A, Crepaldi G and Fioretto P. (2003). Is podocyte injury relevant in diabetic nephropathy? Studies in patients with type 2 diabetes. *Diabetes* 52:1031-1035.

Dalton RN (2010). Serum Creatinine and Glomerular Filtration Rate: Perception and Reality. *Clinical Chemistry* 56:687-689.

Dempsey EC, Newton AC, Rosen MD, Fields AP, Reyland ME, Insel PA and Messing RO. (2000). Protein Kinase C isoenzymes and the regulation of diverse cell responses. *American Journal of Physiology Lung Cell Molecular Physiology* 279: L429-L438.

Diabetes Control and Complications Trial Research Group. (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New England Journal of Medicine.* 329:977-986.

Ding G, Reddy K, Kapasi AA, Franki N, Gibbons N, Kasinath BS and Singhal PC. (2002). Angiotensin II induces apoptosis in rat glomerular epithelial cells. *American Journal of Physiology Renal Physiology* 283: F173-F180

Dirks JH, de Zeeuw D, Agarwal SK, Atkins RC, Correa-Rotter R, D'Amico G, Bennett PH, ElNahas M, Valdes RH, Kaseje D, Katz IJ, Naicker S, Rodriguez-Iturbe B, Schieppati A, Shaheen F, Sitthi –Amorn C, Solez K, Viberti G, Remuzzi G and Weening JJ. (2005). Prevention of chronic kidney disease and vascular disease: toward global health equality: the Bellagio 2004 Declaration. *Kidney International* 98 : S1-S6.

Donnelly R, Yeung JMC and Manning G. (2003). Microalbuminuria: A common, independent cardiovascular risk factor, especially but not exclusively in type 2 diabetes. *Journal of Hypertension* 21: S7-S12.

Dotta F, Censini S, van Harlteren AG, Marselli L, Masini M, Dionisi S, Mosca F, Boggi U, Muda AO, Prato SD, Elliot JF, Covacci A, Rappuoli R, Roep BO and Marchetti P. (2007). Coxsackie B4 virus infection of beta cells and natural killer cell insulinitis in recent-onset type 1 diabetic patients. *Proceedings of the National Academy of Sciences of the United States of America.* 104:5115-5120.

Doublier S, Salvidio G, Lupia E, Ruotsalainen V, Verzola D, Deferrari G and Camussi G. (2003). Nephrin expression is reduced in human diabetic nephropathy: evidence for a distinct role for glycated albumin and angiotensin 11. *Diabetes* 52:1023-1030.

Droge W. (2002). Free radicals in the physiological control of cell function. *Physiology Review* 82:47-95.

Dronavalli S, Duka I and Bakris GL. (2008). The pathogenesis of Diabetic Nephropathy. *Clinical Practice Endocrinology and Metabolism* 4 444-452.

Droumaguet C, Balkau B, Simon D, Caces E, Tichet J, Charles MA,and Eschwege E. (2006). The DESIR Study Group – Use of HbA1 c in Predicting Progression to Diabetes in French Men and Women – Data from an Epidemiological Study on the Insulin Resistance Syndrome (DESIR). *Diabetes Care* 29:1619-1625.

Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J and Brownlee M. (2000). Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and includes plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proceedings of the National Academy of Science of the United States of America* 97:12222-12226.

Durvasula RV, Petermann AT, Hiromura K, Blonski M, Pippin J, Mundel P, Pichler R, Griffin S, Couser WG and Shankland SJ. (2004). Activation of a local tissue angiotensin system in podocytes by mechanical strain. *Kidney International* 65: 30-39.

Durvasula RV and Shankland SJ. (2005). Mechanical strain increases SPARC levels in podocytes: Implications for glomerulosclerosis *American Journal Renal Physiology* 289:F577-F584.

Eisenbarth G. (2004). Prediction of type 1 diabetes: the natural history of the prediabetic period. *In* Immunology of Type 1 Diabetes, 2nd edition. Eisenbarth GS, Ed New York: Springer Publishing, p 268-281, 2004.

El-Agouza I, Abu SA and Sirdah M. (2002). The effect of iron deficiency anaemia on the levels of hemoglobin subtypes: possible consequences for clinical diagnosis. *Clinical and Laboratory Hematology* 24:285-289.

Emmanuel I. Agaba1 EI , Wigwe CM, Agaba PA and Tzamaloukas AH. (2009). Performance of the Cockcroft-Gault and MDRD equations in adult Nigerians with chronic kidney disease. *International Urology and Nephrology* 41:635-642.

Endlich N and Endlich K. (2006). Stretch, tension and adhesion-adaptive mechanisms of the actin cytoskeleton in podocytes. *European Journal of Cell Biology* 85: 229-234.

Faure P, Wiernsperger N, Polge C, Favier A and Halimi S. (2008). Impairment of the antioxidant properties of serum albumin in patients with diabetes: protective effects of metformin. *Clinical Science* 114:251-256.

Feng Y, Wang Q, Wang Y, Yard B and Lang F. (2005). SGK1-mediated Fibronectin formation in Diabetic Nephropathy. *Cellular Physiology and Biochemistry* 16:237-244.

Ficociello LH, Perkins BA, Silva KH, Finkelstein DM, Ignatowska-Switalska H, Gaciong Z, Cupples LA, Aschengrau A, Warram JH and Krowlewski AS. (2007). Determinants of progression from microalbuminuria to proteinuria in patients who have type 1 diabetes and are treated with angiotensin-converting enzyme inhibitors. *Clinical Journal of the American Society of Nephrology* 2:461-469.

Florian JA, Kosky JR, Ainslie K, Pang Z, Dull RO and Tarbell JM. (2003). Heparan sulfate proteoglycan is a mechanosensor on endothelial cells. *Circulation Research* 93:e136-e142.

Fontsefe N, Salinas I, Bonal J, Bayés B, Riba J, Torres F, Rios J, Sanmarti A and Romero R. (2006). Are prediction equations for glomerular filtration rate useful for the long-term monitoring of type 2 diabetic patients. *Nephrology Dialysis and Transplantation* 21:2152-2158.

Forbes JM, Cooper ME, Oldfield MD and Thomas MC. (2003). Role of Advanced Glycation End Products in Diabetic Nephropathy. *Journal of American Society Nephrology* 14:S254-S258.

Forbes JM, Thallas V, Thomas MC, Founds HW, Burns WC, Jerums G and Cooper ME. (2003). The breakdown of pre-existing advanced glycation end products is associated with reduced renal fibrosis in experimental diabetes. *The Federation of American Societies for Experimental Biology Journal* 17:1762-1764.

Freedman BI, Shihabi ZK, Andries L, Cardona CY, Peacock TP, Byers JR, Russell GB, Stratta RJ and Bleyer AJ. (2010). Relationship between Assays of Glycaemia in Diabetic Subjects with Advanced Chronic Kidney Disease. *American Journal of Nephrology*. 31:375-379.

Friess U, Beck-A, Kohne E, Lehmann R, Koch S, Haring HU, Schmuelling RM and Schleicher E. (2003). Novel hemoglobin variant [β66(E10)-Lys→Asn], with decreased oxygen affinity, causes falsely low hemoglobin A_{1c} values by HPLC. *Clinical Chemistry* 49:1412-1415.

Froissart M, Rossert J, Jacquot C, Paillard M and Houillier P. (2005). Predictive performance of the modification of diet in renal disease and Cockcroft-Gault equations for estimating renal function. *Journal of American Society of Nephrology* 16:763-773.

Fukuoka K, Nakao K, Morimoto H, Nakao A, Takatori Y, Arimoto K, Taki M, Wada T and Makino H. (2008). Glycated Albumin levels predict long-term survival in diabetic patients undergoing haemodialysis. *Nephrology* 13:278-283.

Furlong F, Crean J, Thornton L, O'Leary R, Murphy M and Martin F. (2007). Dysregulated intracellular signaling impairs CTGF-stimulated responses in human mesangial cells exposed to high extracellular glucose. *American Journal of Physiology Renal Physiology* 292:1691-1700.

Gallo A, Ceolotto G, Pinton P, Iori E, Murphy E, Rutter GA, Rizzuto R, Semplicini A and Avogaro A. (2005). Metformin prevents glucose-induced protein kinase C-β2 activation in human umbilical vein endothelial cells through an antioxidant mechanism. *Diabetes* 54:1123-1131.

Garg AX, Kiberd BA, Clark WF, Haynes RB and Clase CM. (2002). Albuminuria and renal insufficiency prevalence guides population screening: results from the NHANES III. *Kidney International* 61:2165-2175.

GenWay Biotech, Inc. 6777 Nancy Ridge Drive, San Diego, CA 92121. www.genwaybio.com 2011.

Giorgino F, Laviola L, Cavallo PP, Solnica B, Fuller J and Chaturvedi N. (2004). Factors associated with progression to macroalbuminuria in microalbuminuriac type 1 diabetic patients; The EURODIAB Prospective Complications Study. *Diabetologia* 47:1020-1028.

Giunti S, Barit D and Cooper ME. (2006). Mechanisms of Diabetic Nephropathy Role of Hypertension. *Hypertension.* 48:519.

Gleissner CA, Sanders JM, Nadler J and Ley K. (2008). Upregulation of aldose reductase during foam cell formation as possible link between diabetes, hyperlipidemia and atherosclerosis. *Arteriosclerosis, Thrombosis and Vascular Biology* 28:1137-1143.

Gloyn AL, Pearson ER, Antcliff JF, Proks P, Bruining GJ, Singerland AS, Howard N, Srinivasan S, Silva JM, Molnes J, Edghill EL, Frayling TM, Temple IK, Mackay D, Shield JP, Sumnik Z, van Rhijn A, Wales JK, Clark P, Gorman S, Aisenberg J, Ellard S, Njølstad PR, Ashcroft FM and Hattersley AT. (2004). Activating mutations in the gene encoding the ATP-sensitive potassium-channel subunit Kir6.2 and permanent neonatal diabetes. *New England Journal of Medicine* 350:1838-1849.

Goh SY and Cooper ME. (2008). Clinical review: The role of advanced glycation end products in progression and complications of diabetes. *Journal of Clinical Endocrinology and Metabolism* 93:1143-52.

Goldberg HJ, Whiteside CI and Fantus IG. (2002).The Hexosamine Regulates the Plasminogen Activator Inhibitor-1 Gene Promotor and Sp1 Transcriptional Activation through Protein Kinase C-Bi and –δ*. *The Journal of Biological Chemistry* 277:33833-33841.

Goldberg HJ, Whiteside CI, Hart GW and Fantus IG. (2006). Posttranslational, Reversible O-Glycosylation Is Stimulated by High Glucose and Mediates Plasminogen Activator Inhibitor-1 Gene Expression and Sp1 Transcriptional Activity in Glomerular Mesangial Cells. *Endocrinology* 147:222-231.

Goldin A, Beckman JA, Schmidt AM and Creager MA. (2006). "Advanced glycation end products: sparking the development of diabetic vascular injury". *Circulation* 114:597–605.

Goldstein DE, Little RR, Lorenz RA, Malone JI, Nathan D, Peterson CM, and Sacks DB. (2004). Tests of glycaemia in diabetes. *Diabetes Care* 27:1761-1773.

Gopalakrishnan G and Gourabathini SP. (2007). Marginal Kidney Donor *Journal of Urology* 23:286-293.

Greenbaum CJ, Cuthbertson D, Krischer JP and the Diabetes Prevention Trial of Type 1 Diabetes Study Group. (2001). Type 1 Diabetes Manifested Solely by 2-h Oral Glucose Tolerance Test Criteria. *Diabetes* 50:470-476.

Gu L, Hagiwara S, Fan Q, Tanimoto M, Kobata M, Yamashita M, Nishitani T, Gohda T, Ni Z, Qian J, Horikoshi S and Tomino Y. (2006). Role of receptor for advanced glycation end-products and signaling events in advanced glycation end-product –induced monocyte chemoattractant protein-1 expression in differentiated mouse podocytes. *Nephrology Dialysis Transplantation* 21:299-313.

Guha M, Xu ZG, Tung D, Lanting L and Natatajam R. (2007) Specific down-regulation of connective tissue growth factor attenuates progression of nephropathy in mouse models of type 1 and type 2 diabetes. *The Federation of American Societies for Experimental Biology* 21:3355-3368.

Ha TS, Hong EJ, Ahn EM and Ahn HY. (2009). Regulation of Type IV Collagen α Chains of glomerular epithelial cells in Diabetic conditions. *Journal of Korean Medical Science* 24:837-843.

Hallan S, Asberg A, Lindberg M and Johnsen H. (2004). Validation of the Modification of Diet in Renal Disease formula for estimating GFR with special emphasis on calibration of the serum creatinine assay. *American Journal of Kidney Disease* 44:84-93.

Hanefoldt CD, Pollock JS and Harshfield GA. (2008). Racial differences in Microalbumin Excretion in Healthy Adolescents. *Hypertension* 51;334-338.

Hari PK, Veeranna V, Sudhakar R, Manickam P, Kaur R, Pradhan J, Jacob S and Afonso L. (2010). Does the presence of microalbuminuria porten a worse outcome in non-diabetic patients with metabolic syndrome? *Journal of the American College of Cardiology* 55:A140.E1314.

Haskins K, Bradley B, Powers K, Fadok V, Flores S, Ling X, Pugazhenthi S, Reusch J and Kench J. (2003). Oxidative stress in type 1 diabetes. *Annals of the New York Academy of Sciences* 1005:43-54.

Hattori Y, Suzuki M, Hattori S and Kasai K. (2002). Vascular Smooth Muscle Cell Activation by Glycated Albumin (Amadori Adducts). *Hypertension* 39:22-28.

Hayden MR, Sowers JR and Tyagi SC. (2005). The central role of vascular extracellular matrix and basement membrane remodeling in metabolic syndrome and type 2 diabetes: the matrix preloaded. *Cardiovascular Diabetology* 4:9.

Hein G, Wiegand R, Lehmann G, Stein G and Franke S. (2003). Advanced glycation end-products pentosidine and N-carboxymethyllysine are elevated in serum of patients with osteoporosis. *Rheumatology* 42: 1242-1246.

Hillege HL, Fidler V, Diercks GFH, van Gilst WH, de Zeeuw D, van Veldhuisen DJ, Gans ROB, Janssen WMT, Grobbee DE and do Jong PE ; for the Prevention of Renal and Vascular End Stage Disease (PREVEND) Study Group. (2002). Uinary Albumin Excretion Predicts Cardiovascular and Noncardiovascular Mortality in General Population. *Circulation* 106:1777-1782.

Hovind P, Rossing P, Tarnow L and Parving HH. (2003). Smoking and Progression of Diabetic Nephropathy in Type 1 Diabetes. *Diabetes Care* 26:911-916.

Hovind P, Rossing P, Tarnow L, Schmidt UM and Parving HH. (2001). Progression of diabetic nephropathy. *Kidney International* 59:702-709.

Ibrahim H, Mondress M, Tello A, Fan Y, Koopmeiners J, andThomas W. (2005). An alternative formula to the Cockcroft-Gault and the Modification of Diet in Renal Disease formulas in predicting GFR in individuals with type 1 diabetes. *Journal of the American Society of Nephrology* 16:1051-1060.

Iglesius-de la Cruz, MC, Ziyadeh FN, Isono M, Kouahahou M, Han DC, Kalluri R, Mundel P and Chen S. (2002). Effects of high glucose and TGF-β on the expression of collagen IV and vascular endothelial growth factor in mouse podocyes. *Kidney International* 62:901-913.

Inaba M, Okuno S, Kumeda Y, Yamada S, Imanishi Y, Tabata T, Okamura M, Okada S, Yamakama T, Ishimura E, Nishizawa Y and Osaka CKD Expert Research Group. (2007). Glycated albumin is a better glycaemic indicator than glycated heamoglobin values in hemodialysis patients with diabetes: Effect of Anaemia and Erythropoietin injection. *Journal of the American Society of Nephrology* 18:896-903.

Jeansson M and Granquist AB. Nystrom JS and Haraldsson B. (2006). Functional and molecular alterations of the glomerular barrier in long-term diabetes in mice. *Diabetologia* 49:2200-2209.

Jefferson JA, Shankland SJ and Pichler RH. (2008). Proteinuria in diabetic kidney disease: a mechanistic viewpoint. *Kidney International* 74:22-36.

Jones GRD and Lim EM. (2003). The National Kidney Foundation Guideline on Estimation of the Glomerular Filtration Rate. *Clinical Biochemistry* 24:95-98.

Jung DS, Li JJ, Kwak SJ, Lee SH, Park J, Song YS, Yoo TH, Han SH, Lee JE, Kim DK, Moon SJ, Kim YS, Han DS and Kan SW. (2008). FR167653 Inhibits Fibronectin Expression and Apoptosis in Diabetic Glomeruli and in High Glucose-Stimulated Mesangial Cells. *American Journal of Physiology Renal Physiology* 295:F595-F604.

Kalantar-Zadeh K, Kopple JD, Regidor DL, Jing J, Shinaberger CS, Aronovitz J, McAllister CJ, Whellan D and Sharma K. (2007). A1C and survival in maintenance hemodialysis patients. *Diabetes Care* 30:1049-1055.

Kallner A, Ayling PA and Khatami Z. (2008). Does eGFR improve the diagnostic capability of S-Creatinine concentration results? A retrospective population based study. *International Journal of Medical Science.* 5:9-17.

Kang YS, Park YG, Kim BK, Han SY, Jee YH, Han KH, Lee MH, Song HK, Cha DR,Kang SW and Han DS. (2006). Angiotensin II stimulates the synthesis of vascular endothelial growth factor through the p38 mitogen activated protein kinase pathway in cultured mouse podocytes. *Journal of Molecular Endocrinology* 36:377-388.

Kanters SDJM, Banga JD, Algra A, Frijns RCJM, Beutler JJ and Fijnheer R. (2001). Plasma levels of cellular fibronectin in Diabetes. *Diabetes Care* 24:323-327.

Karumanchi SA, Epstein FH and Stillman IE. (2005) Is loss of podocyte foot processes necessary for the induction of proteinuria. *American Journal of Kidney Disease* 45:436.

Katakami N, Matsuhisa M, Kaneto H, Matsuoka TA, Sakamoto K, Nakatani Y, Ohtoshi K, Okano RH, Kosugi K, Hori H and Yamasaki Y. (2005). Decreased Endogenous Secretory Advanced Glycation End Product Receptor in Type 1 Diabetic Patients Its possible association with diabetic vascular complications. *Diabetes Care* 28: 2716-2721.

Katavetin P, Eiam-Ong S and Suwanwalaikorn S. (2006). Proglitazone reduces urinary protein and urinary transforming growth factor-beta excretion in patients with type 2 diabetes and overt nephropathy. *Journal of the Medical Association of Thailand 89*:170-177.

Kidney Disease Outcomes Quality Initiative (K/DOQI) clinical practice guidelines and clinical practice recommendations for diabetes and chronic kidney disease. (2007). *American Journal of Kidney Disease* 49:S12.

Kilpatrick ES, Rigby AS and Atkin SL. (2008). The relationship between mean glucose and HbA_{1C} in pre-menopausal women compared to males in the Diabetes Control and Complications Trial. *Diabetes Medicine* 25:112-113.

King KD, Jones JD and Warthen J. (2005). Microcascular and Macrovascular Complications of Diabetes Mellitus. *American Journal of Pharmaceutical Education.* 69 Article 87.

Kirk JK, D'Agostino RB, Bell RA, Passmore LV, Bonds DE, Kerter AJ and Venkat Narayan KM. (2006). Disparities in HbA1c Levels Between African-American and Non-Hispanic White Adults with Diabetes. A meta-analysis. *Diabetes Care* 29:2130-2136.

Koka V, Wang W, Huang XR, Mitsuyama SK, Truong LD and Lan HY (2006). Advanced Glycation End Products Activate a Chymase-Dependent Angiotensin II–Generating Pathway in Diabetic Complications. *Circulation* 113:1353-1360.

Konta T, Hao Z, Abiko H, Ishikawa M, Takahashi T, Ikeda A, Ichikawa K, Takasaki S and Kuboa I. (2006). Prevalence and risk factor analysis of microalbuminuria in Japanese general population: The Takahata study. *Kidney International* 70:751-756.

Kosecki SM, Rodgers PT and Adams MB. (2005). Glycemic monitoring in diabetics with sickle cell plus beta-thalassaemia hemoglobinopathy. *Annals Pharmacotherapy* 39:1557-1560.

Kotajima N, Kimura T, Kanda T, Obata K, Kuwabara A, Fukumura Y, and Kobayashi I. (2000). Type IV collagen as an early marker for diabetic nephropathy in non-insulin-dependent diabetes mellitus. *The Journal of Diabetes and its Complication* 14:13-17.

Kouzuma T. (2004). Study of glycated amino acid elimination for an improved enzymatic glycated albumin measurement method. *Clinical Chimica Acta* 346: 135-143.

Kowluru RA and Chan PS. (2007). Oxidative Stress and Diabetic Retinopathy. *Experimental Diabetes Research* 2007. Article ID 43603. doi:10.1155/2007/43603.

Kowluru RA, Abbas SN and Odenbach S. (2004). Reversal of hyperglycaemia and diabetic nephropathy: effect of reinstitution of good metabolic control on oxidative stress in the kidney of diabetic rats. *Journal of Diabetes and its Complications* 18:282-288.

Kowluru RA. (2005). Effect of advanced glycation end products on accelerated apoptosis of retinal capillary cells under in vitro conditions. *Life Sciences* 76:1051-1060.

Koya D and King GL. (1998). Protein kinase C activation and the development of diabetic complications. *Diabetes* 47:859-866.

Lam S, van der Geest RN, Verhagen NAM, Daha MR and van Kooten C. (2004). Secretion of collagen type IV by human renal fibroblasts is increased by high glucose via a TGF-ß-independent pathway. *Nephrology Dialysis and Transplantation* 19:1694-1701.

Lamb EJ, Tomson CR and Roderick PJ. (2005). Estimating kidney function in adults using formulae. *Annals of Clinical Biochemistry* 42:321-345.

Lassègue B and Clempus R.E. (2003). Vascular NAD(P)H oxidases: specific features, expression, and regulation. *American Journal of Physiology-Regulatory Interactive and comparative Physiology* 285: R277-R297.

LeBleu VS, MacDonald and Kalluri R. (2007). Structure and Function of Basement Membranes. *Experimental Biology Medicine* 232:1121-1129.

Lee HB, Yu MR, Yang Y, Jian Z and Ha H. (2003). Reactive oxygen species-regulated signalling pathways in diabetic nephropathy. *Journal of the American Society of Nephrology* 14:S241-S245.

Lee HS, Moon KC, Song CY, Kim BC, Wang S and Hong HK. (2004). Glycated albumin activates PAI-1 transcription through Smad DNA binding sites in mesangial cells. Articles in Press. *American Journal of Physiology Renal Physiology* doi.10.1152/ajprenal.00034.2004.

Levey AS, Andreoli SP, DuBose T, Provenzano R and Collins AJ. (2007). Chronic Kidney Disease: Common, Harmful, and Treatable – World Kidney Day 2007. *Journal of the American Society of Nephrology 2*:401-405.

Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N and Roth D. (1999). A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Annals of Internal Medicine* 130:461-470.

Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, Hogg RJ, Perrone RD, Lau J, and Eknoyan G. (2003). National Kidney Foundation practice guidelines for chronic kidney disease : evaluation, classification, and stratification. *Annals of Internal Medicine* 139:137-147.

Levey AS, Eckhart KU, Tsukamoto Y, Levin A, Coresh J, Rossert J, De Zeeuw D, Hotsetter TH, Lamaeire N and Ekonoyan G. (2005). Definition and classification of chronic kidney disease: a positional statement from Kidney disease: Improving Global Outcomes (KDIGO). *Kidney International* 67:2089-2100.

Levey AS, Greene T, Kusek JW, Beck GJ and Group MS. (2000). A simplified equation to predict glomerular filtration rate from serum creatinine. *Journal of American Society of Nephrology* 11:A0828.

Lewis J, Agodoa L, Cheek D, Greene T, Middleton J, O'Connor D, Ojo A, Phillips R, Sika M, and Wright J Jr; African-American Study of Hypertension and Kidney Disease. (2001). Comparison of cross-sectional renal function measurements in African Americans with hypertensive nephrosclerosis and of primary formulas to estimate glomerular filtration rate. *American Journal of Kidney Disease* 38:744-753. [Erratum *American Journal of Kidney Disease* 39:444, 2002].

Li and Wang S. (2010). Glycated albumin up-regulates upstream stimulatory factor 2 gene transcription in mesangial cells. *American Journal of Physiology- Renal Physiology* (April, 2010). doi.10.1152/ajprenal.00074.2010.

Lin K, Knight E, Hogan ML and Singh AK. (2003). A comparison of predicting equations for estimating glomerular filtration rate in adults without kidney disease. *Journal of American Society of Nephrology* 14:2573-2580. [Erratum *Journal of American Society of Nephrology* 16:2814

Liu X, Luo F, Pan K, Wu W and Chen H. (2007). High glucose upregulateds connective tissue growth factor exopression in human vascular smooth muscle cells. *Bio Medical Central Cell Biology* 8:1 doi:10.1186/1471-2121-8-1.

Londono I and Bendayan M. (2001). Temporary effects of circulating Amadori products n glomerular filtration properties in the normal mouse. *American Journal of Renal Physiology* 280:F103-F111.

Londono I and Bendayan M. (2005). Glomerular handling of native albumin in the presence of circulating modified albumins by the normal rat kidney. *American Journal of Physiology Renal Physiology* 289:F1201-F1209.

Lutale JJK, Thordarson H, Abbas ZG and Vetvik K. (2007). Microalbuminuria among Type 1 and Type 2 diabetic patients of African origin in Dar Es Salaam, Tanzania. *Bio Medical Central Nephrology* 8: doi:10.1186/1471-2369-8-2.

Ma Y-C, Zuo L, Chen J-H, Luo Q, Yu X-Q, Li Y, Xu J-S, Huang S-M, Wang L-N, Huang W, Wang M, Xu G-B, and Wang H-Y, on behalf of the Chinese eGFR Investigation Collaboration: (2006). Modified glomerular filtration rate estimating equation for Chinese patients with chronic kidney disease. *Journal of the American Society of Nephrology* 17: 2937-2944.

Ma YC, Zuo L, Zhang CL, Wang M, Wang RF and Wang HY. (2007). Comparison of 99mTc-DTPA renal dynamic imaging with modified MDRD equation for glomerular filtration rate estimation in Chinese patients in different stages of chronic kidney disease. *Nephrology Dialysis and Transplant* 22: 417–423.

MacIsaac RJ, Panagiotopoulos S, McNeil KJ, Smith TJ, Tsalamandris C, Hao H, Matthews PG, Thomas MC, Power DA and Jerums G. (2006). Is nonalbuminuric renal insufficiency in type 2 diabetes related to an increase in intrarenal vascular disease? *Diabetes Care* 29: 1560–1566.

MacIsaac, RJ, Tsalamandris, C, Thomas, MC E. Premaratne, S. Panagiotopoulos, T. J. Smith, Poon, M.A. Jenkins, S.I. Ratnaike, D.A. Power and G. Jerums. (2006). Estimating glomerular filtration rate in diabetes: a comparison of cystatin-C- and creatinine-based methods. *Diabetologia* 49:1686-1689.

Mao Y and Schwarzbauer JE. (2005). Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. Matrix biology. *Journal of the International Society for Matrix Biology* 24:389-399.

Mason RM and Wahab NA. (2003). Extracellular matrix metabolism in diabetic nephropathy. *Journal of the American Society of Nephrology* 14 :1358 –1373.

Mason RM. (2009). Connective tissue growth factor (CCN2), a pathogenic factor in diabetic nephropathy. What does it do? How does it do it? *Journal of Cell Communication and Signalling* 3:95-104.

McFarlane SI and Sowers JR. (2003). Aldosterone Function in Diabetes Mellitus: Effects on Cardiovascular and Renal Disease. *The Journal of Clinical Endocrinology and Metabolism* 88:516-523.

McKee KK, Capizzi S and Yurchenco PD. (2009). Scaffold-forming and Adhesive Contributions of Synthetic Laminin-binding Proteins to Basement Membrane Assembly. *Journal of Biological Chemistry* 284:8984-8994.

Mocan MC, Kadayifcilar S and Eldem B. (2006). Elevated intravitreal interleukin-6 levels in patients with poliferative diabetic retinopathy. *Canadian Journal of Ophthalmology* 41:747-752.

Mogensen CE. (2003). Microalbuminuria and hypertension with focus on type 1 and type 2 diabetes. *Journal of Internal Medicine* 254:45-66.

Mohan V. (2007). Macrovascular Component of Diabetes Atherosclerosis and Insulin (CUPS-18). *Journal of Associations of Physicians of India* 55:3-8.

Mora C and Navarro JF. (2006). Inflammation and diabetic nephropathy. *Current Diabetes Reports* 6:463-468.

Moriya T, Groppoli TJ, Kim Y and Mauer M. (2001). Quantitative immunoelectron microscopy of type IV collagen in glomerular of type I diabetic patients. *Kidney International* 59:317-323.

Motala AA, Omar MAK and Pirie FJ. (2003). Epidemiology of Type 1 and Type 2 Diabetes in Africa. *Journal Of Cardiovascular Risk* 10: 77-83.

Motala AA, Pirie FJ, Gouws E, Amod A and Omar MAK. (2001). Microvascular complications in South African patients with long standing duration of diabetes mellitus. *South African Medical Journal* 91:987-992.

Murdaugha LS, Dillonb J and Gaillard ER. (2009). Modifications to the basement membrane protein laminin using glycolaldehyde and A2E: A model for aging in Bruch's membrane. *Experimental Eye Research* 89:187-192.

Muttar D, Subratty A, Jowaheer V and Joonus N. (2010). Analyzing Anthropometry and Metabolic Variables Associated With Microalbumin and C-Reactive Protein as Markers of Early Glomerular Dysfunction Among Mauritian Patients Suffering from Type 2 Diabetes. *The Internet Journal of Laboratory Medicine* 4:1937-8181.

Myers GL, Miller WG, Coresh J, Fleming J, Greenberg N, Greene T, Hotstetter T, Levey AS, Panteghini M, Welch M and Eckfeldt JH for the Working Group of the National Kidney Disease Education Program. (2006). Recommendations for the Improving Serum Creatinine Measurement: A Report from the Laboratory Working Group of the National Kidney Disease Education Program. *Clinical Chemistry* 52:5-18.

Nagayama H, Inaba M, Okabe R, Emoto M, Ishimura E, Okazaki S and Nishizawa Y. (2009). Glycated albumin as an improved indicator of glycemic control in hemodialysis patients with type 2 diabetes based on fasting plasma glucose and oral glucose tolerance test. *Biomedicine and Pharmacotherapy* 63: 236-240.

Nangaku M, Inagi R, Miyata T and Fujita T. (2008). Hypoxia and Hypoxia-Inducible Factor in Renal Disease. *Nephron Experimental Nephrology* 2008:110:e1-e7. doi.10.1159/000148256.

Navarro JF and Mora C. (2005). Role of Inflammation in diabetic complications. *Nephrology Dialysis and Transplantation* 16:1537-1538.

Navarro JF and Mora C. (2008). The Role of Inflammatory Cytokines in Diabetic Nephropathy. *Journal of the American Society of Nephrology* 19:433-442.

Nayak BS and Roberts L. (2006). Relationship between inflammatory markers, metabolic and anthropometric variables in the Carribbean type 2 diabetic patients with and without microvascular complications. *Journal of Inflammation* 3:17.

Nguyen TQ, Tranow L, Jorsal A, Oliver N, Roestenberg P, Ito Y, Parving HH, Rossing P, van Nieuwenhoven FA and Goldschmeding R. (2008). Plasma connective tissue growth factor is an independent predictor of end-stage renal disease and mortality in type 1 diabetic nephropathy. *Diabetes Care* 31:1177-1182.

Nishikawa T, Edelstein D and Brownlee M. (2000). The missing link: a single unifying mechanism of diabetic complications. *Kidney International* 58:S26-S33.

Obrosova I.G, Minchenko A,G, Vasupuram R, White L, Abatan OI, Kumagai AK, Frank RN and Stevens MJ. (2003). Aldose reductase inhibitor fidarestat prevents retinal oxidative stress and vascular endothelial growth factor over-expression in streptozotocin-diabetic rats. *Diabetes* 52:864-871.

Obrosova IG, Pacher P, Szabó, Zsuzsanna Z, Hirooka H, Stevens MJ and Yorek MA. (2005). Aldose reductase inhibitioncounteracts odidative-nitrosative stress and poly(ADP-ribose) polymerase activation in tissue sites for diabetes complications. *Diabetes* 54:234-242.

Ochodnicky P, Henning RH, van Dokkum RPE and de Zeeuw D. (2006). Microalbuminuria and endothelial dysfunction: Emerging targets for primary prevention of end-organ damage. *Journal of Cardiovascular Pharmacology* 47:S151-S162.

Okada H, Kikuta T, Kobayashi T, Inoue T, Kanno Y, Takigawa M, Sugaya T, Kopp JB and Suzuki H. (2005). Connective tissue growth factor expressed in tubular epithelium plays a pivotal role in renal fibrogenesis. *Journal of the American Society of Nephrology* 16:133-143.

Okada T, Nakao T, Matsumoto H, Shino T, Nagaoka Y, Tomaru R and Wada T. (2007). Association between markers of glycaemic control, cardiovascular complications and survival in type 2 diabetic patients with end-stage renal failure. *Internal Medicine* 46:807-814.

Paavonen AS, Watson AMD, Li J, Paavonen K, Koitka A, Calkin AC, Barit D, Coughlan MT, Drew BG, Lancaster GI, Thomas M, Forbes JM, Nawroth PP, Bierhaus A, Cooper ME and Dahm KAG. (2008). Receptor for Advanced Glycation End Products (RAGE) Deficiency Attenuates the Development of Atherosclerosis in Diabetes. *Diabetes* 57: 2461–2469.

Pankov R and Yamada KM. (2002). Fibronectin at a glance. *Journal of Cell science* 115:3861-3863.

Pantsulaia T. (2006). Role of TGF-beta in pathogenesis of diabetic nephropathy. *Georgian Medical News* 131:13–18.

Paroni R, Ceriotti F, Galanello R.Battista LG, Scurati E, Paleari R, Chemello L, Quaino V, Scaldaferri L, Lapolla A and Mosca A. (2007). Performance characteristics and clinical utility of an enzymatic method for the measurement of glycated albumin in plasma. *Clinical Biochemistry* 40:1398-1405.

Peacock TP, Shihabi ZK, Bleyer AJ, Dolbare EL, Byers JR, Knovich MA, Calles-Escandon J, Russell GB and Freedman BI. (2008). Comparison of glycated albumin and hemoglobin A1C levels in diabetic subjects on hemodialysis. *Kidney International* 73:1062-1068.

Perkins BA, Ficociello LH, Roshan B, Warram JH and Krolewski AS. (2010). In patients with type 1 diabetes and new-onset microalbuminuria the development of advanced chronic kidney disease may not require progression to proteinuria. *Kidney International* 77:57-64.

Perkins, LH. Ficociello, BE. Ostrander, KH. Silva, J W, Warram JH., and Krolewski AS. (2007). Microalbuminuria and the Risk for Early Progressive Renal Function Decline in Type 1 Diabetes. *American Society of Nephrology* 18:1353-1361.

Peters AM, Bird NJ, Halsall I, Peters C and Mitchell AR. (2009). Evaluation of the modification of diet in renal disease equation (eGFR) against simultaneous, dual marker multi-sample measurements of glomerular filtration rate. *Annals of Clinical Biochemistry* 46:58-64.

Petrash J.M. (2004). All in the family: Aldose reductase and closely related aldo-keto reductases. *Cell and Molecular Life Sciences* 61:737-749.

Pickup JC (2004). Inflammation and activated innate immune system in the pathogenesis of type 2 diabetes. *Diabetes Care* 27:813-823.

Pociot F and McDermott MF. (2002). Genetics of type 1 diabetes mellitus. *Genes Immunology* 3:235-249.

Poge U, Gerhardt T, Stoffel-Wagner B, Palmedo H, Sauerbruch T and Woitas RP. (2006). Prediction of glomerular filtration rate in renal transplant recipients: cystatin C or modification of diet in renal disease equation? *Clinical Transplantation* 20:200-205.

Poggio ED, Wang X, Greene T, Van Lente F, Dennis VW and Hall PM. (2005). Performance of the modification of diet in renal disease and Cockcroft-Gault equations in the estimation of GFR in health and in chronic kidney disease. *Journal of American Society of Nephrology* 16:459-466.

Pösch E, Schlötzer-Schrehardt U, Brachvogel B, Saito K, Ninomiya Y and Mayer U. (2003). Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. *Development* 131:1619-1628.

Pozzi A, Zent R, Chetyrkin S, Borza C, Bulus N, Chaung P, Chen D, Hudson B and Voziyan P. (2009). Modification of Collagen IV by Glucose or Methylglyoxal Alters Distinct Mesangial Cells Function. *Journal of the American Society of Nephrology* 20:2119-2125.

Pozzilli P and Di Mario U. (2001). Autoimmune Diabetes Not requiring Insulin at Diagnosis (Latent Autoimmune Diabetes of the Adult). Definition, characterization, and potential prevention. *Diabetes Care* 24:1460-1467.

Premaratne E, MacIsaacs RJ, Finch S, Panagiotopoulos S, Ekinci E and Jerums G. (2008). Serial measurements of cystatin C are more accurate than creatinine-based methods in detecting declining renal function in type 1 Diabetes. *Diabetes Care* 31:971-973.

Pricci F, Leto G, Amadio L, Lacobini C, Cordone S, Catalano S, Zicari A, Sorcini M, Di Mario U, and Pugliese G. (2003). Oxidative stress in diabetic-induced endothelial dysfunction involvement of nitric oxide and protein kinase C. *Free Radical Biology and Medicine* 35:683-694.

Pu LJ, Lu L, Shen WF, Zhang Q, Zhang RY, Zhang JS, Hu J, Yang ZK, Ding FH, Chen QJ, Shen J, Fang DH and Lou S. (2007). Increased serum glycated albumin level is associated with the presence and severity of coronory artery disease in type 2 diabetic patients. *Circulation* 71:1067-1073.

Raghu K. (2003). Basement membranes: structures, assembly and role in tumor angiogenesis. *Nature Reviews Cancer* 3:422-433.

Rask-Madsen C and King GL. (2005). Proatherosclerotic Mechanisms Involving Protein Kinase C in Diabetes and Insulin Resistance. *Arteriosclerosis, Thrombosis, and Vascular Biology* 25:487.

Remuzzi G, Schieppati A and Ruggenenti P. (2002). Clinical practice: nephropathy in patients with type 2 diabetes. *New England Journal of Medicine* 346:1145-1151.

Rewers M, Norris J and Dabelea D. (2004). Epidemiology of Diabetes. *In* Immunology of Type 1 Diabetes, 2^{ND} Edition. Eisenbarth GS, Ed. New York, Springer, p 221-233.

Rich SS. (2006). Genetics of diabetes and its complications. *Journal of the American Society of Nephrology* 17:353-360.

Rienstra H, Katta K, Celie JWAM, van Goor H, Navis G, van den Born J and Hillebrands JL. (2010). Differential Expression of Proteoglycans in Tissue Remodeling and Lymphangiogenesis after Experimental Renal Transplantation in Rats. PLoS ONE 5:e9095. doi:10.1371/journal.pone.0009095.

Rigalleau V, Lasseur C, Perlemoine C, Barthe N, Raffaitin C, Liu C, Chauveau P, Baillet-Blanco L, Beauvieux MC, Combe C and Gin H. (2005). Estimating of Glomerular Filtration Rate in Diabetic Subjects. Cockcroft formula or Modification of Diet in Renal Disease study. *Diabetes Care* 28:838-843.

Roberfroid MB. (2007). Inulin and Oligofructose: Health Benefits and Claims-A Critical Review. Inulin-Type Fructans: Functional Food Ingredients1,2. *The Journal of Nutrition* 137:2493S-2502S

Rohlfing C. (2000). Use of GHb (HbA_{1c}) in screening for undiagnosed diabetes in the US population. *Diabetes Care* 23:187-191.

Rohlfing CL, Wiedmeyer HM, Little RR, England JD, Tenill A and Goldstein DE. (2002). Defining the relationship between plasma glucose and HbA_{1c}: analysis of glucose profiles and HbA_{1c} in the Diabetes Control and Complications Trials. *Diabetes Care* 25:275-278.

Romunstad S, Holmen J, Kvenild K, Hallan H and Ellekjaer H. (2003). Microalbuminuria and all-cause mortality in 2,089 apparently healthy individuals: a 4.4 year follow-up study: the Nord-Trondelag Health Study (HUNT), Norway. *American Journal of Kidney Disease* 42:466-473.

Rossing P, Parving HH and de Zeeuw D. (2006). Renoprotection by blocking the RAAS in diabetic nephropathy—fact or fiction? *Nephrology Dialysis and Transplantation* 21:2354-2357.

Ruggenenti P, Fassi A, Ilieva AP, Bruno S, Iliev IP, Brusegan V, Rubis N, Gherardi G, Arnoldi F, Ganeva M, Ene-Iordache B, Gaspari F, Perna A, Bossi A, Trevisan R, Dodesini AR and Remuzzi G for the Bergamo Nephrologic Diabetes Complications Trial (BENEDICT) Investigators. (2004). Preventing Microalbuminuria in Type 2 Diabetes. *The New England Journal of Medicine* 351:1941-1951.

Rule AD, Larson TS, Bergstrath EJ, Slezak JM, Jacobsen SJ and Coslo FG. (2004). Using serum creatinine to estimate glomerular filtration rate: accurracy in good health and in chronic kidney disease. *Annals of Internal Medicine* 141:929-937.

Salazar R, Brandt R, and Krantz S. (2001). Binding of Amadori glucose-modified albumin by the monocytic cell line MonoMac 6 activates protein kinase C epsilon protein tyrosine kinases and the transcription factors AP-1 and NF-kappa B. *Glycoconjugate Journal* 18:769-777.

Sanna-Cherchi S, Carnevalli ML, Martorana D, Cravedi P, Maggiore U, Alinovi R, Bovino A, Mattei S, Orlandini G, Gatti R, Savi M, Sado Y, Neri TM and Allegri L. (2007). Alterations of type IV Collagen α chains in Patients with Chronic Acquired Glomerulonephropathies: mRNA levels, Protein Expression and Urinary Loss. *American Journal of Nephrology* 27:129-137.

Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, McCullough PA, Kasiske BL, Kelepouris E, Klag MJ, Parfrey P, Pfeffer M, Raij L, Spinosa DJ and Wilson PW. (2003). Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. *Hypertension* 42:1050-1065.

Schaefer L, Raslik I, Gröne HJ, Schönherr E, Macakova K, Ugorcakova J, Budny S, Schaefer RM and Kresse H. (2001). Small proteoglycans in human diabetic nephropathy: Discrepancy between glomerular expression and protein accumulation of decorin, biglycan, lumican, and fibromodulin 1. *The Federation of American Societies of Experimental Biology Journal of the United States of America* 15:559-561.

Schalkwikj CG, Stehouwer CDA and van Hinsberg VWM. (2004). Fructose-mediated non-enzymatic glycation: sweet coupling or bad modification. *Diabetes Metabolism Research and Reviews* 20:369-382.

Schiel R. (2003) Improvements of the quality of diabetes control and decrease in the concentration of AGE-products in patients with type-1 and insulin-treated type 2 diabetes mellitus studies over a period of 10-years (JEVIN). *Journal of Diabetes and its complications* 17:90-97.

Schram MT, Schalkwijk CG and Bootsma AH. (2005). Advanced glycation end products are associated with pulse pressure in type 1 diabetes: the EURODIAB Prospective Complications Study. *Hypertension* 46:232-237.

Shen J, Fang DH and Shen WF. (2006). Value of serum glycated albumin and high-sensitivity C-reactive protein levels in the prediction of presence of coronary artery disease in patients with type 2 diabetes. *Cardiovascular Diabetology* 5:27. doi:10.1186/1475-2840-5-27.

Shui HA, Ka SM, Lin JC, Lee JH, Jin JS, Lin YF, Sheu LF and Chen A. (2006). Fibronectin in blood invokes the development of focal segmental Glomerulosclerosis in mouse model. *Nephrology Dialysis Transplantation* 21:1794-1802.

Singh DK, Winocour P and Farrington K. (2008). Mechanisms of disease: the hypoxia tubular hypothesis of diabetic nephropathy. *Nature Clinical Practice Nephrology* 4:216-226.

Singh N, Armstrong DG and Lipsky BA. (2005). Preventing foot ulcers in patients with diabetes. *Journal of the American Medical Association* 293:217-28.

Sirwal IA, Banday KA, Reshni R, Bhat MA and Wani MM. (2004). Estimation of Glomerular Filtration Rate (GFR). *Journal of Medical Education and Research* 6:121-123.

Sjostrom P, Tidman M and Jones I. (2005). Determination of the production rate and non-renal clearance of cystatin C and estimation of the glomerular filtration rate from serum concentration of cystatin C in humans. *Scandinavian Journal of Clinical Laboratory Investigations* 65:111-124.

Slentz CA, Duscha BD, Johnson JL, Ketchum K, Aiken LB, Samsa GP, Houmard JA, Bales CW and Kraus WE. (2004). Effects of the amount of exercise on body weight, body composition, and measures of central obesity: STRRIDE – A rabdomised controlled study. *Archives of Internal Medicine* 164:31-39.

Stanley CM, Wang Y, Pal S, Kelbe RJ, Harkless LB, Xu X, Chen Z and Steffensen B. (2008). Fibronectin-Fragmentation is a feature of both periodontal disease sites and diabetic foot and leg wounds and modifies cell behaviour. *Journal of Periodontology* 79:861-875.

Steinke JM, Sinaiko AR, Kramer MS, Suissa S, Chavers BM, and Mauer M. (2005). The Early Natural History of Nephropathy in Type 1 Diabetes III. Predictors of 5-Year Urinary Albumin Excretion Rate Patterns in Initially Normoalbuminuric Patients. *Diabetes* 54:2164-2170.

Stevens LA and Levey AS. (2005). Chronic kidney disease in the elderly: How to assess risk. *New England Journal of Medicine* 352:2122-2124.

Stevens LA and Levey AS. (2009). Current Status and Future Perspectives for CKD Testing. *American Journal of Kidney Disease* 53:S17-26.

Stevens LA, Coresh J, Greene T and Levey AS. (2006). Assessing kidney function-measured and estimated glomerular filtration rate. *New England Journal of Medicine* 354:2473-2483.

Stoppacciaro A, Pietrucci A, Fofi C, Raffa S, Torrisi MR and Mené P. (2008). Fibronectin glomerulopathy: an uncommon cause of nephrotic syndrome in systemic lupus erythematosus. *Nephrology Dialysis Transplantation* Plus 4:225-227.

Takeuchi M, Iwaki M, Takino J, Shirai H, Kawakami M, Bucala R and Yamgishi S. (2010). Immunological detection of fructose-derived advanced glycation end products. *Laboratory Investigation* doi:10.1038/labinvest.2010.62.

Tan KC, Chow WS and Ai VH. (2002). Advanced glycation end products and endothelial dysfunction in type 2 diabetes. *Diabetes Care* 25:1055-1059.

Tashiro K, Koyanagi I, Ohara I, Ito T, Saitoh A, Horikoshi S and Tomino Y. (2004). Levels of urinary matrix metalloproteinase-9 (MMP-9) and renal injuries in patients with type 2 diabetic nephropathy. *Journal of Clinical Laboratory Analysis* 18:206-210.

Thomas MC and Atkins R. (2009). Assessment and management of hypertension in patients with type 2 diabetes. *Internal Medicine Journal* 39:143–149.

Thorpe SR and Baynes JW. (2003). Maillard reaction products in tissue protein: a new products and new perspectives. *Amino Acids* 25:275-281.

Tidman M, Sjöström P and Jones I. (2008). A comparison of GFR estimating formulae based upon s-cystatin C and s-creatinine and a combination of the two. *Nephrology Dialysis Transplantation* 23:154-160.

Toth C, Rong LL, Yang C, Martinez J, Song F, Ramji N, Brussee V, Liu W, Durand J, Nguyen MD, Schmidt AM and Zochodne DW. (2008). Receptor for Advanced Glycation End Products (RAGEs) and Experimental Diabetic Neuropathy. *Diabetes* 57:1002-1017.

Tuttle KR, Johnson EC, Cooney SK, Anderberg RJ, Johnson EK, Clifton GD and Meek RL. (2005). Aminoacids injure mesangial cells by advanced glycation end products, oxidative stress and protein kinase C. *Kidney International* 67:953-968.

Tuttle KR. (2005). Linking metabolism and immunology: Diabetic nephropathy is an inflammatory disease. *Journal of the American Society of Nephrology* 16:1537-1538.

United Kingdom Prospective Diabetes Study (UKPDS) Group. (1998). Intensive blood-glucose control with sulfonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 352:837-853.

United Kingdom Prospective Diabetes Study Group. (1998). Tight blood pressure control and risk of microvascular and macrovascular complications in type 2 diabetes: UKPDS *British Medical Journal* 38:703-713.

Unnikrishnan R, Rema M, Pradeepa R, Deepa M, Shanthirani CS, Deepa R and Mohan V. (2007). Prevalence and Risk factors of Diabetic Nephropathy in an Urban South Indian Population. The Chennai Urban Rural Epidemiological Study (CURES 45). *Diabetes Care* 30:2019-2024.

Ulrich, P and Cerami, A. (2001) Protein glycation, diabetes, and aging. *Recent Progress in Hormone Research* 56:1–21.

Uribarri J, Stirban A, Sanders D, Cal W, Negreen M, Beuting CE, Koschinsky T and Vlassara H. (2007). Single Oral Challenge by Advanced Glycation End Products Acutely Impairs Endothelial Function in Diabetic and Nondiabetic Subjects. *Diabetes Care* 30:2579-2582.

Vahma R. (2005). Diabetic Retinopathy - Challenges and future Directions. *American Journal of Ophthalmology* 539-541.

van Deventer HE, George JA, Paiker JE, Becker PJ and Katz IJ. (2008). Estimating Glomerular Filtration Rate in Black South Africans by use of the Modification of Diet in Renal Disease and Cockcroft-Gault Equations. *Clinical Chemistry* 54:1197-1202.

Verhave JC, Fesler P, Ribstein J, du Cailar G and Mimran A. (2005). Estimation of renal function in subjects with normal serum creatinine levels. *American Journal of Kidney Disease* 46:233-241.

Vinik AI, Erbas T, Stasberry KB and Pittenger GL. (2001). Small fiber neuropathy and neurovascular disturbances in diabetes mellitus. *Experimental and Clinical Endocrinology and Diabetes* 109:S451-S473.

Wahab NA, Schaefer L, Weston BS, Yiannikouris O, Wright A, Babelova A, Schaefer R and Mason RM. (2005). Glomerular expression of thrombospondin-1, transforming growth factor β and connective tissue growth factor at diferent stages of diabetic nephropathy and their interdependent roles in mesangial response to diabetic stimuli. *Diabetologia* 48:2650-2660.

Wahab NA, Yevdokimova N, Weston BS, Roberts T, Li XJ, Brinkman H and Mason RM. (2001). Role of connective tissue growth factor in the pathogenesis of diabetic nephropathy. *Biochemistry Journal* 359:77-87.

Wang Q, Wang Y, Minto AW, Wang J, Shi Q, Li X and Quigg RJ. (2008). MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy. *The Journal of the Federation of American Societies for Experimental Biology* 22:4126-1235.

Wang X, Shaw S, Amiri F, Eaton DC and Marrero MB. (2002). Inhibition of the KAK/STAT Signalling Pathways Prevents the Hight Glucose-Induced Increase in TGF-ß and Fibronectin Synthesis in Mesangial Cells. *Diabetes* 51:3505-3509.

Watanabe H, Sanada H and Shigetomi S (2000). Urinary excretion of type IV collagen as a specific indicator of the progression of diabetic nephropathy. *Nephron* 86:27-35.

Way KJ, Katai N and King GL. (2001). Protein kinase C and the development of diabetic vascular complicatoons. *Diabetes Medicine* 18:945-956.

Weigert C, Brodbeck K, Brosius FC III, Huber M, Lehmann R, Friess U, Facchin S, Aulwurm S, Häring HU, Schleicher ED and Heilig CW. (2003). Evidence for a Novel TGF-β1-Independent Mechanism of Fibronectin Production in Mesangial Cells Overexpressing Glucose Transporters. *Diabetes* 52:527-535.

Weiner DE, Tighiouart H, Stark PC, Amin MG, MacLeod B, Griffith JL, Salem DN, Levey AS and Sarnak MJ. (2004). Chronic kidney disease as a risk factor for cardiovascular disease and all cause mortality: a pooled analysis of community-based studies. *Journal of the American Society of Nephrology* 15:1307-1315.

Wells L, Vosslle K and Hart GW. (2001). Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc. *Science* 291:2376-2378.

Wells L, Vosslle K and Hart GW. (2001). Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc. *Science* 291:2376-2378.

Weston BS, Wahab NA and Mason RM. (2003). CTGF Mediates TGF-β-Induced Fibronectin Matrix Deposition by upregulating active α5β1 Integrin in Human Mesangial Cells. *Journal of the American Society of Nephrology* 14:601-610.

Whiteside C, Wang H, Xia L, Munk S, Goldberg HJ and Fantus IG. (2009). Rosiglitazone prevents high-glucose induced vascular endothelial growth factor and collagen IV expression in cultured mesangial cells. *Experimental Diabetes Research* Article ID 910783. doi:10.1155/2009/910783.

Williams CM, Engler AJ, Sloane RD, Galante LL and Schwarzbauer JE. (2008). Fibronectin expresion modulates mammary epithelial cell proliferation during acinar differentiation. *Cancer Research* 68:3185-3192.

Williams MD and Nadler JL. (2007). Inflammatory mechanisms of diabetic complications. *Current Diabetes Reports* 7:242–248.

Wolf G and Ziyadeh. (2007). Cellular and molecular mechanism of proteinuria in diabetic nephropathy. *Nephron Physiology* 106:26-31.

Wolf G, Chen S and Ziyadeh FN. (2005). Perspectives in Diabetes from the periphery of the glomerular capillary wall toward the centre of Disease. Podocyte Injury comes of Age in Diabetic Nephroapthy. *Diabetes* 54:626-1634.

Yoshioka K, Tegoshi H, Ogata M and Yokoo S. (2004). Relationship between increased excretion of Type IV collagen and Plasma brain Natriuretic Peptide levels in Diabetic Nephropathy. *Matsushita Medical Journal* 43:9-13.

Yoshiuchi K, Matsuhisa M, Katakami N, Nakatani Y, Sakamoto K, Matsuoka T, Umayahara Y, Kosugi K, Kaneto H, Yamasaki Y and Hori M. (2008). Glycated Albumin is a Better Indicator for Glucose Excursion than Glycated Hemoglobin in Type 1 and Type 2 Diabetes. *Endocrine Journal* 55:503-507.

Yung S, Lee CYY, Zhang Q and Lau SK. (2006). Elevated gluocse induction of thromobspondidn-1 up-regulates fibronectin synthesis in proximal renal tubular epithelial cells through TFG-β dependent and TFG-β independent pathways. *Nephrology Dialysis Transplantation* 21:1504-1513.

Zhou G, Li C and Cai L. (2004). Advanced Glycation End-Products Induce Connective Tissue Growth Factor-Mediated Renal Fibrosis Predominantly through Transforming Growth Factor ß-Independent Pathway. *American Journal of Pathology* 165:2033-2043.

Ziyadeh FN, Hoffman BB, Han DC, Iglesias-De La Criz MC, Hong SW, Isono M, Chen S, McGowan TA and Sharma K. (2000). Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix gene expansion by treatment with monoclonal antitransforming growth factor-beta antibody in *db/db* diabetic mice. *Proceedings of the National Academy of Sciences of the United States of America* 97:8015-8020.

Ziyadeh FN. (2004). Mediators of Diabetic Renal Disease: The Case for TGF-β as the Major Mediator. *Journal of the American Medical Society of Nephrology* 15:S55-S57.

Zuo L, Ma YC, Zhou YH, Wang M, Xu GB and Wang HY. (2005). Application of GFR-estimating equations in Chinese patients with chronic kidney disease. *American Journal of Kidney Disease* 45:463-472.

APPENDICES

Appendix 1: Glycation study- Informed consent

Glycation study – Informed consent **Study number** [ ]

You, as a person with type 1 diabetes, are invited to participate in a clinical study to assess factors resulting in kidney disease in type 1 diabetes. The study aims to examine the role of proteins in the blood, that have been altered by the addition of glucose (sugar), in the development of kidney disease. The theory that will be assessed is that alteration of blood proteins by the addition of glucose, results in a change in the kidney membranes that ultimately lead to kidney disease. Your participation is entirely voluntary and will neither positively or negatively influence your attendance at the Diabetes Clinic. Should you agree to participate, there will be no change to your therapy and you will be asked to continue to strive for optimal diabetes control. Blood and urine specimens will be drawn at entry into the study and after 6 months, 1 year and 2 years. It is hoped that there will be 170 participants in the study. The samples will be taken at the same time as the routine tests that are done when you attend the clinic. An extra 4 tubes of blood and an extra urine specimen will be needed on these dates. There will be no payment for these specimens. Should you wish to discontinue your participation and resume regular follow up, you are free to do so without any negative consequences on your future management. The data collected pertaining to you will be treated as strictly confidential. The study will hopefully provide further knowledge in the understanding of kidney disease in type 1 diabetes such that effective management strategies can be devised. Any results from the study that have impact on the management of your condition will be given to you directly. This study has approval from the Nelson R Mandela School of Medicine Ethics Committee.

Wena,njengomuntu onesifo sika shukela osijovelayo (type 1 diabetes) uyamenywa ukuba ungenele ucwaningo lokuhlola nokuthola ezinye zezinto (factors) ezibangela isifo sezinso kulesisifo sikashukela. Injongo yalolucwaningo ukuhlola nokuthola indima edlalwa izakhamzimba (proteins) asegazini aseshintshwe ukubakhona kukashukela ekubangeleni isifo sezinso. Umqondo okuzohlolelwa\phezu kwawo wukuthi ukushintsha kwezakhamzimba ngokubakhona kukashukela kwenza kubekhona ushintsho. Ontwentwesini (membrane) lwezinso okuyikona okugcina kubeyimbangela yesifo sezinso. Awuphoqekile ukungenela lolucwaningo noma uyavuma noma awuvumi akuzuphazamiseka indlela ohamba ngayo iDiabetic Clinic. Uma uvuma ngeke lubekhona ushintsho emithini oyidlayo, uyocelwa nje ukuba uqhubeke nokuzama ukuthi ushukela uhlale usezingeni elamukelekayo. Ekuqaleni kocwaningo kuzothathwa amagazi nomchamo kubuye kuphindwe emuva kwezinyanga eziyisithupha (6 months). Emuva ko nyaka (1 year) nasemva kweminyaka emibili (2 years). Silindele ukuba sibe nabantu abangu 170 kulolucwaningo. Amagazi nomchamo kuzothathwa njengoba ehlala, ethathwa uma uze eClinic. Sizothatha amabhodlela (tubes) amane nomchamo owodwa ngaphezulu. Ngezinsuku kuthathwa amagazi nomchamo. Akukho imali ezokhokhelwa ucwaningo. Ungaphuma noma inini uma ungasathandi ukuqhubeka nocwaningo futhi ukuphuma kwakho akuzukwenza ukuba kubekhona ukungaphatheki kwakho kahle ekliniki. Imininingwane etholakele ngawe izoba yimfihlo engedalulwe. Siyethembake ukuthi lolucwaningo luzosinika ulwazi olungcono ngesifo sezinso nokuzama izindlela ezingcono zokusisebenza. Imiphumela yalolucwaningo ephathelene nokwelashwa kwakho izonikwa wena ngqo. Lolucwaningo lungemvume ye Nelson R Mandela School of Medicine Ethics Committee.

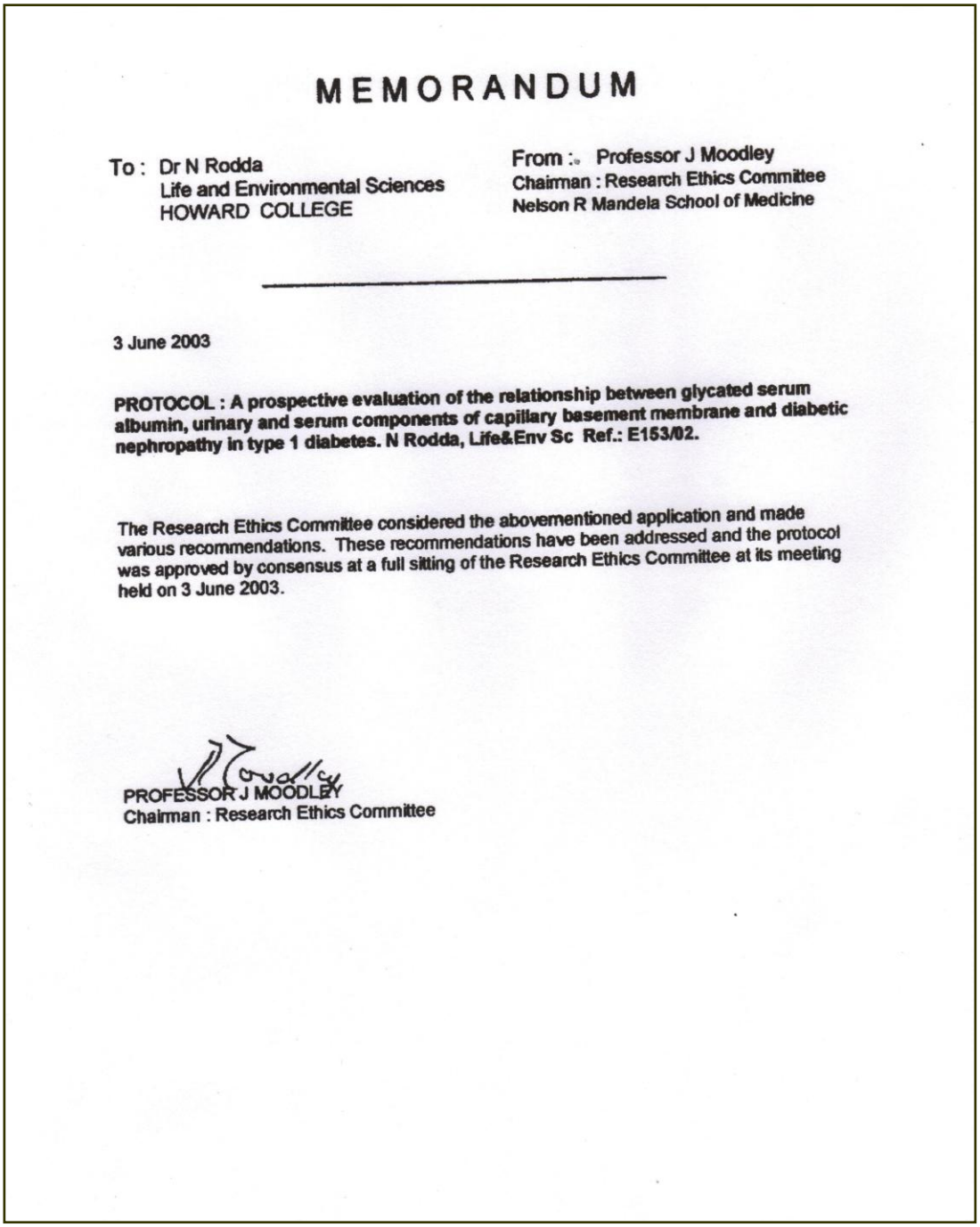
Signed:_____ Date:_____

Printed name:_____

Witness:_____ Date:_____

Printed name:_____

Appendix 2: Ethics approval letter



MEMORANDUM

To : Dr N Rodda
Life and Environmental Sciences
HOWARD COLLEGE

From : Professor J Moodley
Chairman : Research Ethics Committee
Nelson R Mandela School of Medicine

3 June 2003

PROTOCOL : A prospective evaluation of the relationship between glycated serum albumin, urinary and serum components of capillary basement membrane and diabetic nephropathy in type 1 diabetes. N Rodda, Life&Env Sc Ref.: E153/02.

The Research Ethics Committee considered the abovementioned application and made various recommendations. These recommendations have been addressed and the protocol was approved by consensus at a full sitting of the Research Ethics Committee at its meeting held on 3 June 2003.

PROFESSOR J MOODLEY
Chairman : Research Ethics Committee

THE MEASUREMENT OF GLOMERULAR BASEMENT MEMBRANE COMPONENTS AND GLYCATED ALBUMIN AS IMPROVED MARKERS OF INCIPIENT DIABETIC NEPHROPATHY

by

Anban Naidoo

Submitted in fulfilment of the academic requirements

for the degree Master of Science

Department of Biological and Conservation Sciences

Faculty of Science

University of KwaZulu-Natal

Durban

June 2010

Abstract

Diabetes causes early structural changes to the glomerular basement membrane (GBM), which alters its function and leads to loss of protein in urine. Formation of advanced glycation endproducts (AGEs) is one mechanism proposed to be responsible for the structural changes to the GBM. AGEs are thought to affect blood flow *i.e.* glomerular filtration rate (GFR) and vascular permeability which over time manifests as overt proteinuria. The gradual loss of minute amounts of protein (albumin) is referred to as microalbuminuria (MA). Microalbuminuria is a dynamic process, with patients regressing to normoalbuminuria more often than progressing to overt proteinuria. Microalbuminuria is not specific to patients with diabetic nephropathy (DN) and new markers specific to DN are being sought. A prospective study was undertaken at the Inkosi Albert Luthuli Central Hospital (IALCH) to evaluate the relationship of serum glycated albumin, urinary and serum components of capillary basement membrane and DN in South African Black and Indian patients with type 1 diabetes. The study was undertaken with sampling of blood and urine at baseline, 6-months, 1 year and 2-year follow-up. Serum glycated albumin, urinary type IV collagen and plasma fibronectin were measured at each visit. Since correlations could be performed only at each time point individually, generalised estimating equation (GEE) regression models were constructed in SPSS (15.0) with time specified as a factor in order to take account of relationships among variables over time. The results of this study showed that serum percentage glycated albumin (PGA), plasma fibronectin (FN) and urinary type IV collagen were not better predictors of incipient impaired renal function than MA. Although previous authors have variously reported serum GA, plasma FN and urinary type IV collagen to be predictive of impaired renal function, these studies were conducted mainly in patients with overt DN. The present study suggest that markers of overt renal dysfunction are not necessarily useful predictors of incipient DN. Differences in predictive relationships point to a different disease processes in the two ethnic groups. Of particular note was the lack of a predictive relationship of either fasting plasma glucose (FPG) or glycated haemoglobin (HbA_{1c}) with any of isotope GFR, estimated GFR and proteinuria in Black patients. The most significant finding of this study showed that combination of serum creatinine and MA provided broadest range of predictors of isotope GFR, estimated GFR and proteinuria.

Preface

The experimental work described in this dissertation was carried out in the Department of Biological and Conservation Sciences at the University of KwaZulu-Natal, Durban, under the supervision of Dr Nicola Rodda and Dr Fraser Pirie.

These studies represent original work by the author, and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

……

A. Naidoo
June 2010

DECLARATION 1 - PLAGIARISM

I, ……. declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced
   b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: ……

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3-DG	3-Deoxyglucosone
ACE	Angiotensin Converting Enzyme
AGEs	advanced glycation end-products
ANG(II)	Angiotensin II
BMI	Body mass index
CHD	Coronary Heart Disease
COX	cycloxygenase
CTGF	Connective tissue growth factor
CVD	Cardiovascular disease
DAG	diacylglycerol
DBP	diastolic blood pressure
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
DN	Diabetic nephropathy
ECM	Extracellular matrix
ESRD	end-stage renal disease
ETC	Electron transport chain
FPG	fasting plasma glucose
GFR	glomerular filtration rate
GMC	glomerular mesangial cells
GSH	gluthathione peroxidase
HDL	high density lipoprotein
IAA	Insulin auto antibodies
ICA	Islet cell antibodies
ICAM-1	Intracellular adhesion molecule 1
IDDM	Insulin Dependent Diabetes Mellitus
IL-6	interleukin-6
K/DOQI	Kidney Disease Outcome Quality Initiative
LDL	Low-density lipoprotein
MAP	Mitogen-Activated Protein Kinase
MA	Microalbuminuria

MCP-1	Monocyte chemotactic protein-1
MnSOD	Manganese Superoxide Dismutase
NADH	Nicotinamide adeninedinucleotide
NAD/NADPH	Nicotinamide adenine dinucleotide phosphate/ reduced nicotinamide adenine dinucleotide
NIDDK	National Institute of Diabetes and Digestive and Kidney Disease
OGTT	Oral glucose tolerance test
OD	optical density
PAD	Peripheral Artery Disease
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet-derived Growth Factor
PKC	Protein kinase C
RAGE	receptor for advanced glycation endproducts
ROS	Reactive oxygen species
SBP	systolic blood pressure
sICAM	Soluble Intracellular Adhesion Molecule
Sp1	Specificity protein 1
sVCAM	Soluble Vascular Cell Adhesion Molecule
TCA	Tricarboxylic acid
TGF-β	Transforming growth factor –β
TNF-α	Tumor necrosis factor-α
UAE	Urinary Albumin Excretion
UCPs	Uncoupling proteins
UKPDS	United Kingdom Prospective Diabetes Study
VCAM	vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

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Dr R. Parboosing, Department of Virology, NHLS, IALCH

To all my dear friends, God has truly blessed me with your presence in my life. I thank you for always supporting me and being a friend to me.

To all the staff of Dr Bouwer and Partners, Ampath. I thank you all for the friendship, support and encouragement over the years. To the Management of Dr Bouwer and Partners, I am truly indebted for all the support I received while managing Kingsway Laboratory.

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To all present and past staff members and students of the Department of Biological and Conservation Sciences, thank you for your advice, encouragement, support and friendship.

This project would not have been possible without the financial support of the Medical Research Council South Africa, National Research Foundation (NRF).

National Health Laboratory Services (NHLS), Department of Chemical Pathology, Inkosi Albert Luthuli Central Hospital, Academic Complex, Catomanor , Durban ,4001.

Department of Nuclear Medicine, Inkois Albert Luthuli Central Hospital, Cato Manor , Durban ,4001.

ETHICS

This study was given full ethics approval by the Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Medical Research Ethics Committee, Ref.: EI53/02.

Dedication

To my parents, Mr Subrayalu Naidoo and Mrs Kamlaveni Naidoo. I thank you for being there all my life and supporting me through all of life's perils. To my father, I thank you for all the lessons you have taught me in life and making me wise. To my mother, no words could express my love for you. You have been the greatest inspiration in this life and I thank you with all my soul for your unselfish love. God bless you both.

To my grandparents, Mr Karnan Muthusamy Pillay and Mrs Sithama Pillay. I am honoured to be your grandson. You have both been my support, inspiration and guidance through this life. No grandchild could ask for more.

To my dear aunt, Selverani Muthusamy. I thank you with all my heart for always being there for me. Your love, kindness, friendship and support through this life, I truly appreciate.

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To my dear brother, Agilan Naidoo. I thank you for your friendship, love and fun times we shared. Thanks for all the artwork. I wish you well in all you do.

To Mahendra Bipraj, Chandrani and Aruni. I am truly blessed with your presence.

To that Ancient being, ruler of my soul,
make me worship you unendingly…