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Chapter 2

Bio-Chemical Aspects, Pathophysiology of Microalbuminuria and Glycated Hemoglobin in Type 2 Diabetes Mellitus

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Additional information is available at the end of the chapter

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1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia and derangement in protein and fat metabolism [1]. The worldwide prevalence of diabetes was approximately 2.8% in 2000 and is estimated to grow to 4.4% by 2030. Approximately 40% of patients with type 1 diabetes and 5 - 15% of patients with type 2 diabetes eventually develop end stage renal disease (ESRD), although the incidence is substantially higher in certain ethnic groups [2, 3]. The main risk factors for the development of diabetes are ethnic variations, changes in the food habits, obesity and altered lifestyles. However in type 2 diabetic patient additional factors, related or unrelated to diabetes plays an important role in causation of diabetic nephropathy such as hypertension, dyslipidemia, obesity and it has been named as metabolic syndrome [4]. There are mainly three types of diabetes which include Type 1 diabetes, Type 2 diabetes including a related condition called pre-diabetes and gestational diabetes. The occurrence of diabetic nephropathy varies with type of diabetes and highest risk indiviusuals are type 1 diabetics, but also type 2 diabetics have significant risk. The studies have shown that incidence of renal failure in type 1diabetes may be decreasing due to better preventive measures. However the incidence of renal complications in type 2 diabetes showed uprising [5-8] because type 2 diabetes accounts for at least 90% of all patients with diabetes. Thereby number of type 2 patients with nephropathy and ESRD exceeds those with type 1 diabetes overall.

Diabetic nephropathy is one of the most serious complication of diabetes and the most common cause of end stage renal disease. Advanced diabetic nephropathy is also the leading cause of glomerulosclerosis and end-stage renal disease worldwide. 20% to 40% of

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patients with diabetes ultimately develop nephropathy, although the reason why not all patients with diabetes develop this complication is unknown [9]. The combination of hypertension and diabetes is an especially dangerous clinical situation; both are risk factors as singly or in combination for micro vascular and macro vascular complications of diabetes and for diabetes-related mortality. It's unfortunate that most of diabetics at the time of diagnosis will have hypertension and studies have shown that 50% of patients with diabetes and hypertension results in a sevenfold increase in mortality [10]. Concomitant nephropathy in patients with diabetes and hypertension results in a 37-fold increase in mortality.

The main treatment dialysis and renal transplantation are costly [11, 12] and most of the poor patients cannot afford the same. Patients with type 2 diabetes undergoing maintenance dialysis require significantly higher financial resources than those suffering from nondiabetic end-stage renal diseases. Furthermore, this group of patients has a very poor prognosis on maintenance dialysis owing to extremely high mortality due to various cardiovascular events [13].

Is the diabetic nephropathy preventable, the answer is yes as diabetic nephropathy progresses from subclinical disease, through the earliest clinically detectable stage characterized by microalbuminuria i.e., urinary albumin 30 to 300mg/day to overt nephropathy with macroalbuminuria [14-16].The combination of strict glycemic control and various biochemical parameters in the form of microalbuminuria, glycated hemoglobin have decreased the occurrence of nephropathy.

Various sensitive tests are available to identify patients with renal involvement early in the clinical course and clinicians should have the knowledge about diabetic nephropathy in the form of its onset, prevention, progression, and treatment in their patients.

Detection of microalbuminuria identifies not only individuals who are at risk of developing renal diseases [(17, 18] but also cardiovascular events and death [19] in these patients. Up to 30% of people with newly diagnosed type 2 diabetes will already have abnormally high urine albumin levels I.e. macroalbuminuria which indicates that many may have overt diabetic nephropathy at the time of diagnosis.

Renal disease is strongly linked to heart disease and the presence of microalbuminuria is a predictor of worse outcomes for both in renal and cardiac patients. Microalbuminuria does not directly cause cardiovascular events; it serves as a marker for identifying those who may be at increased risk. Microalbuminuria is caused by glomerular capillary injury and so may be a marker for diffuse endothelial dysfunction. According to Steno hypothesis, albuminuria might reflect a general vascular dysfunction and leakage of albumin and other plasma macromolecules such as low density lipoproteins into the vessel wall that may lead to inflammatory responses and in turn start the atherosclerotic process [20, 21].

Recently, it has been suggested that microalbuminuria may be a risk factor for the development of cardiovascular disease in non-diabetics and may therefore have a role in screening programs [22].

Early detection of nephropathy through screening of diabetic patients allows early intervention and better control of progression of nephropathy and cardiovascular events and mortality.

1.1. Socio-economic burden of diabetes in India

Type 2 diabetes is the commonest form of diabetes constituting 90% of the diabetic population in any country and prevalence of diabetes is estimated to increase from 4% in 1995 to 5.4% by the year 2025 (23). The countries with the largest number of diabetic subjects are India, China and U.S. and in the former two countries diabetes occurs mostly in the age range of 45-64yrs, in contrast with an age of >65 in the developed countries. Epidemiological studies conducted in India showed that not only was the prevalence high in urban India but it was also increasing [24-26]. This is mainly attributed to life style changes and genetic predisposition in Indian population.

The period between1989-95 showed a 40% rise in the prevalence and subsequently a further increase of 16.4% was seen in the next 5 years. A national survey of diabetes conducted in six major cities in India in the year 2000 showed that the prevalence of diabetes in urban adults was 12.1%. The prevalence of impaired glucose tolerance (IGT) was also high (14.0%). A younger age at onset of diabetes had been noted in Asian Indians in several studies [26, 27].

In the national study, onset of diabetes occurred before the age of 50 years in 54.1% of cases, implying that these subjects developed diabetes in the most productive years of their life and had a greater chance of developing the chronic complications of diabetes. The recent studies found that the occurrence of diabetic nephropathy with respect to age is been decreasing and most of people affected in early ages.

Table 1 shows the prevalence of the vascular complications observed in a study by the Diabetes Research Centre [28].

Microvascular		Macrovascular	
Retinopathy	23.7	Cardiovascular disease	11.4
Background	20.0	Peripheral vascular disease	4.0
Proliferative	3.7	Cerebrovascular accidents	0.9
Nephropathy	5.5	Hypertension	38.0
Polyneuropathy	27.5		

Table 1. Prevalence (%) of vascular complications in type 2 diabetes

Prevalence of retinopathy is high among the Indian type 2 diabetic subjects. Another study done in 1996 in South India showed a prevalence of 34.1% of retinopathy [29]. The prevalence of nephropathy in India was less (8.9% in Vellore) [30]. 5.5% in Chennai [28] when compared with the prevalence of 22.3% in Asian Indians in the UK in the study by Samanta et al in 1991[31].

1.2. The main health problems related to diabetes are

Diabetes can have a significant impact on quality of life by increasing risk for a variety of complications mainly long standing. These include:

Chronic complications
Blindness(Mainly cataract and retinopathy)
Renal Disease
Hypertension
Cardiac Disease and Stroke
Amputations
Nervous System Disease
Pregnancy complications

Table 2. Main chronic complications in diabetes

2. Diabetic nephropathy

The abnormal glycemic status of diabetes is closely related to the development of micro-vascular complications. However, in humans the evidence of a straightforward causal relationship between hyperglycemia and renal disease is less compelling than in animal models. The development of diabetic nephropathy is characterized by a progressive increase in the excretion of albumin, continued increase in blood pressure and decline in glomerular function which later leads to end stage renal failure. The patients with diabetes are more prone for this condition due to associated factors like hyperlipidemia and hypertension. The mortality and morbidity is high and it's mainly due to a cardiovascular event [32, 33].

There are various factors which lead to diabetic nephropathy like biochemical, hormonal, immunological and rheological.

- Biochemical factors include long standing hyperglycemia and glycosylation process [34].
- Studies have shown that growth hormone promotes basement membrane thickening in diabetes [35].
- Both exogenous and endogenous insulin autoantibodies, IAA contributed in basement membrane thickening [36].
- The red blood cell deformity due to glycosylation and fibrin deposition results in altered permeability and hypercoagulability in diabetic patients [37].

2.1. Genetic and ethnic role

Although we know that all patients with diabetes will not develop ESRD this is due to the good glycemic and blood pressure control. In addition to the risks of poor glycemic control and hypertension, a subset of patients may be at greater risk for nephropathy based on inherited factors. Familial clustering of patients with nephropathy may result from similarly

poor glycemic or blood pressure control or may have additional independent genetic basis [38, 39].

Diabetic siblings of patients with diabetes and renal disease are five times more likely to develop nephropathy than diabetic siblings of diabetic patients without renal disease. Even this has been proved by histo-pathological studies in twins with type 1 diabetics [40, 41]. Genetic factors may play an important role in diabetic nephropathy and/or may be clustered with genes influencing other cardiovascular diseases. There is ongoing research in identifying genetic loci for diabetic nephropathy susceptibility through genomic screening and candidate gene approaches [42-44]. A recent genome scan for diabetic nephropathy in African Americans identified susceptibility loci on chromosomes 3q, 7p and 18q [45] and in Pima Indians it has been identified on chromosome 7 [46].

Diabetic nephropathy and hypertension are multifactorial disorders resulting from both environmental and genetic factors, which make it complex and difficult to identify at the genetic level what confers susceptibility to diabetic kidney disease. Gene polymorphism play's an important role for example in renin–angiotensin system, nitric oxide (NO), aldose reductase, glucose transporter 1 (GLUT-1), and lipoproteins which are potentially involved in the genetic predisposition to hypertension, vascular reactivity, and insulin resistance [47].

A recent study has shown that the strong association between a polymorphism in the 5′-end of the aldose reductase gene and the development of diabetic nephropathy in type 1 diabetic patients [47].

ESRD is known to be more prevalent in certain ethnic groups—Native Americans, Mexican Americans, and African Americans—than in Caucasian Americans. Certainly, there is reason for special vigilance for early signs of nephropathy in these high-risk populations, whose members presumably have a genetic predisposition to nephropathy.

The factors which contribute for the development of diabetic nephropathy are shown in Table 3.

Metabolic factors	• Advanced glycation end products (AGEs) • Aldose reductase (AR)/ Polyol pathway
Hemodynamic factors	• Angiotensin 2 / renin – angiotensin system (RAS) • Endothelin • Nitric oxide
Intracellular factors	• Diacyglycerol (DAG) – protein kinase C (PKC) pathway
Growth factors and cytokines	• Transforming growth factor β (TGF- β) • Growth hormone (GH) and insulin –like growth • Factors (IGFs) • Vascular endothelial growth factor (VEGF) • Platelet-derived growth factor (PDGF)

Table 3. Factors involved in development of diabetic nephropathy

2.2. Natural history of diabetic nephropathy (Table 3) and renal changes in diabetic nephropathy

Diabetic nephropathy is a spectrum of progressive renal lesions secondary to diabetes mellitus ranging from renal hyper-filtration to end stage renal disease. The earliest clinical evidence of nephropathy is the presence of microalbuminuria. It occurs in 30% of type 1 diabetics 5 to 15 years after diagnosis but may be present at diagnosis in type 2 diabetics as the time of onset of type 2 diabetes is often unknown. The microalbuminuria progresses to overt proteinuria over the next 7 to 10 years. Once overt proteinuria develops, renal function progressively declines and end stage renal disease is reached after about 10 years.

Stage 1	• Glomerular hypertension and hyper filtration • Normoalbuminuria: urinary albumin excretion rate (AER) <20 μg/min • Raised GFR, normal serum creatinine
Stage 2	• "Silent phase" (structural changes on biopsy but no clinical manifestations) • Normoalbuminuria
Stage 3	• Microalbuminuria: AER 20 – 200μg/min • Normal serum creatinine • Increased blood pressure
Stage 4	• Overt "dipstick positive" proteinuria (macroalbuminuria) : AER > 200μg/min • Hypertension • Serum creatinine may be normal Increase in serum creatinine with progression of nephropathy
Stage 5	• End stage renal failure • Requiring dialysis or transplant to maintain life

Adapted from SIGN Guidelines (48)

Table 4. Evolution of diabetic renal disease

Renal changes are characterized by specific renal morphological and functional alterations which include:

- Features of early diabetic changes in the form of glomerular hyper filtration, glomerular and renal hypertrophy, increased urinary albumin excretion (UAER).
- Increased basement membrane thickness (BMT) and mesangial expansion with the accumulation of extracellular matrix (ECM) proteins such as collagen, fibronectin and laminin.
- Advanced diabetic nephropathy is characterized by proteinuria, a decline in renal function, decreasing creatinine clearance, glomerulosclerosis and interstitial fibrosis.

3. Pathophysiology of microalbuminuria

Normal human urine contains only very small quantities of albumin, less than 30 mg of albumin being excreted by healthy adults in 24 hours. The appearance of large amounts of

albumin in the urine is a cardinal sign of renal damage, especially glomerular disease, and is not detectable by screening techniques using urinary dipsticks.

Various studies have shown different factors play a role in microalbuminuria. The two important factors plays a role in urinary albumin excretion are trans glomerular passage of albumin and tubular reabsorption. The glomerular and tubular proteinuria can be distinguished by simultaneously measuring the urinary β_2-microglobulin and albumin [49, 50].

Rodicio et.al., in their article has put forward the causes of microalbuminuria in hypertension which is invariably associated with diabetes as follows: -

- Can be a consequence of an augmented intraglomerular capillary pressure.
- Reflects the existence of intrinsic glomerular damage leading to changes in the glomerular barrier filtration.
- May be the result of a tubular dysfunction in normal reabsorption of filtered albumin.
- It may be the renal manifestation of a generalized, genetically conditioned vascular endothelial dysfunction which may therefore link urinary albumin excretion and elevated risk of cardiovascular diseases [51].

3.1. Structural abnormalities seen during increased excretion of albumin

There is a general belief that increased urine albumin excretion in diabetic nephropathy is mostly glomerular in origin. For albumin to appear in the urine it must cross the glomerular filtration barrier, which consists of fenestrated glomerular endothelial cells, the glomerular basement membrane, and glomerular epithelial cell or podocyte.

It has been seen that increased intraglomerular pressure, loss of negatively charged glycosaminoglycan's in the basement membrane and, later, increased basement membrane pore size, all contribute to the albuminuria. The earliest morphological change of diabetic nephropathy is expansion of the mesangial area [52] and is caused by an increase in extracellular matrix deposition and mesangial cell hypertrophy. After a short period of proliferation, mesangial cells exposed to hyperglycemia become arrested in the G1-phase of the cell cycle and is mediated by p27 Kip1, an inhibitor of cyclin-dependent kinases [53, 54].

Hyperglycemia activates the mitogen-activated protein kinases (MAPKs) which lead to a post-transcriptional increase in p27 Kip1 expression [55].

In addition, ANG II further enhances p27 Kip1 induction and blockade of ANG II attenuates high glucose mediated mesangial cell hypertrophy [54]. Thickening of the GBM is progressive over years; both increased extracellular matrix synthesis and impaired removal contribute to GBM thickening.

There is a decrease in the expression of heparin sulphate and the extent of sulphation followed by increase in collagen type IV deposition. The type of collagen expressed in GBM mainly contains α 3, α 4, and α 5 chains and mesangial matrix has α 1 and α 2 of type IV collagen and increased expression is seen in diabetic populations [56, 57].

The recent evidence shows that an alteration in structure and function of podocytes occurs early in diabetic nephropathy. The podocytes which are adhering to GBM through integrin's are altered due to hyperglycemia.

In addition, renal biopsies from Pima Indians showed a broadening in podocyte foot processes and a concomitant reduction in the number of podocytes per glomerulus [58] in type 2 diabetic patients.

The structural abnormalities seen are:

Mesangial expansion	Fibrin cap lesion
Glomerulosclerosis (diffuse, nodular)	
Basement membrane thickening (glomerular and tubular)	Endothelial foam cells
Arteriosclerosis	Tubular atrophy
Capsular drop lesion	Interstitial fibrosis
Interstitial inflammation	Podocyte abnormalities

Table 5. Main structural abnormalities in diabetic nephropathy

3.2. Glomerular and tubular mechanisms

The alterations in glomerular function and tubular reabsorption play an important role in microalbuminuria. The glomeruli receive 25% of cardiac output per day. Of the 70kg of albumin that passes through the kidneys every 24hr, less than 0.01% reaches the glomerular ultra filtrate and hence enters the renal tubules [59, 60, and 61]. Almost all filtered albumin is reabsorbed by proximal tubule via a high affinity, low capacity endocytic mechanism with only 10-30mg/24hour appearing in the urine [62].

The passage of albumin through glomeruli depends on two main factors, charge and size. The negative charge on the glomerular membrane repels the anionic proteins thereby preventing the passage of albumin molecules through glomeruli normally. The loss of glomerular charge selectivity has been found in both diabetics and non-diabetic population with microalbuminuria [63, 64].

Established microscopic abnormalities include thickening of the glomerular basement membrane, accumulation of mesangial matrix, and increase in the numbers of mesangial cells with disease progression there is a close relationship between mesangial expansion and declining glomerular filtration [65].

Mesangial expansion also correlates inversely with capillary filtration surface area, which itself correlates with glomerular filtration rate. Changes in the tubulointerstitium, including thickening of tubular basement membrane, tubular atrophy, interstitial fibrosis and arteriosclerosis, have been well described. Interstitial enlargement correlates with glomerular filtration, albuminuria, and mesangial expansion. It has been suggested that the accumulation of protein in the cytoplasm of proximal tubular cells causes an inflammatory reaction which leads to tubulointerstitial lesions [65]. Similarly, rise in blood pressure plays an important role by altering the fraction of plasma filtered by the glomerulus.

3.3. Changes in endothelial function

Increased systemic capillary permeability has also been linked with microalbuminuria in healthy populations and recent study shows that endothelial dysfunction leads to impaired insulin action as well as to capillary leakage of albumin [67, 68].

Therefore, microalbuminuria may be a marker of generalized vascular disease, as the formation of atherosclerotic thrombi is related to endothelial dysfunction in arteries. Thus in addition to being an early marker of incipient diabetic nephropathy, urinary albumin excretion may represent common pathways for the development of both large and small vessel disease making microalbuminuria as a possible marker for cardiovascular diseases.

3.4. Cellular and molecular mechanisms

Abnormalities of many cellular processes have been described in the kidney cells of experimental and/or human diabetes. Most work so far has been focused on the glomerular endothelial and mesangial cells. Direct effects of hyperglycemia per se (glucose toxicity), glycation, formation of advanced glycation products, increased flux through the polyol and hexosamine pathways have all been implicated in the pathogenesis of diabetic nephropathy.

Recently it has been suggested that the central abnormality linking all of these pathways is oxidative stress, a defect in the mitochondrial electron transport chain resulting in over-production of reactive oxygen molecules which stimulate each of the above pathways [69].

Increased activity of a large number of growth factors has been demonstrated in diabetes [70].

- Transforming growth factor ß-1 and connective tissue growth factor: May be involved in the fibrotic changes seen in mesangium and interstitium.
- Growth hormone and insulin like growth factor-1 (IGF-1) appear to be associated with the glomerular hyper filtration and hypertrophy.
- Vascular endothelial growth factor (Synthesized by the podocyte): Plays a major role in maintaining the fenestrae in glomerular endothelial cells, has pressor effects leading to constriction of the efferent glomerular arterioles.
- Glucose itself also stimulates some signaling molecules, leading to the increased intra glomerular pressure. Several isoforms of protein kinase C, diacyl glycerol, mitogenic kinases, and transcription factors are all activated in diabetic nephropathy.

3.5. Hemodynamic abnormalities

The glomerular hemodynamic changes in the form of hyper filtration and hyper perfusion results in decreased resistance in both afferent and efferent arterioles of the glomerulus. Many diverse factors including prostanoids, nitrogen oxide (NO), atrial natriuretic factor, growth hormone, glucagon, insulin, angiotensin II (ANG II), and others have been

implicated as agents causing hyperperfusion and hyper filtration [71].Hyperglycemia itself stimulates the synthesis of angiotensin II, which leads to various hemodynamic changes in the form of trophic, inflammatory and profibrogenic effects.

The vascular endothelial growth factors (VEGFs), and cytokines, such as transforming growth factor β (TGF-β), may mediate hyper filtration by dilatation of the afferent vessels by inhibiting calcium transients [72]. Furthermore, TGF- β increases NO production in early diabetes, probably by up-regulation of endothelial NO synthase (eNOS) mRNA expression and by enhancing arginine resynthesis [72]. Thus, TGF- β could clearly play a role in diabetic vascular dysfunction [74].

The studies have shown that shear stress and mechanical strain causes hemodynamic alterations by inducing the autocrine and/or paracrine release of cytokines and growth factors.

The factors contributed are:

- Renin-angiotensin system
- Vasoactive hormones such as nitric oxide, prostacyclin, Endothelin -1,Urotensin

4. Role of glycated hemoglobin in diabetes

Glycated hemoglobin (HbA1c), a marker of average glycaemia, is a predictor of micro vascular complications in diabetic individuals. However, it is not yet clear whether the HbA1c is an indicator of the risk of the macro vascular complications associated with diabetes mellitus.

HbA1c is the product of non-enzymatic reaction between glucose and free amino groups of hemoglobin. This reaction, called glycosylation, involves lots of other proteins, too and it is the principal mechanism through which glucotoxicity occurs. Other mechanism involved s is: oxidative stress, activation of the polyols pathway, activation of protein kinase-C, endothelial damage, hemodynamic and coagulative changes [75].

HbA1c reflects average plasma glucose over the previous 8 to 12 weeks as the life span of RBC's is 80-120days [76]. It can be performed at any time of the day and does not require any special preparation such as fasting. These properties have made it the preferred test for assessing glycemic control in diabetics. More recently, there has been substantial interest in using it as a diagnostic test for diabetes and as a screening test for persons at high risk of diabetes [77]. The use of HbA1c can avoid the problem of day-to-day variability of glucose values, and importantly it avoids the need for the person to fast and to have preceding dietary preparations. These advantages have implications for early identification and treatment which have been strongly advocated in recent years.

However, HbA_{1c} may be affected by a variety of genetic, hematologic and illness-related factors [78]. The most common important factors worldwide affecting HbA_{1c} levels are hemoglobinopathies (depending on the assay method employed), certain anemia's, and disorders associated with accelerated red cell turnover such as malaria [79].

Long term prospective studies are required in all major ethnic groups to establish more precisely the glucose and HbA1c levels predictive of micro vascular and macro vascular complications. A working group should be established to examine all aspects of HbA1c and glucose measurement methodology.

The diagnosis of diabetes in an asymptomatic person should not be made on the basis of a single abnormal plasma glucose or HbA1c value. At least one additional HbA1c or plasma glucose test result with a value in the diabetic range is required, fasting, a random (casual) sample, or the oral glucose tolerance test (OGTT) report.

The main long term vascular complications are coronary artery disease, stroke, renal failure etc. The measurement of glycosylated hemoglobin (GHb) is one of the well-established means of monitoring glycemic control in patients with diabetes mellitus [80]. In 1968 Bookchin and Gallop subsequently reported that the largest of these minor fractions, designated HbA_{1c}, had a hexose moiety linked to the N-terminus of the β-globin chain [81].The functions of many proteins depend upon post translational modification, hemoglobin is one such protein [82]. Hemoglobin (Hb) is composed of four globin chains and adult hemoglobin (HbA) is the most abundant form in most adults and consists of two α and two β chains. Fetal hemoglobin (HbF), which is predominantly present at birth, consists of two α and two γ chains. HbF is a minor form in normal adults. HbA_2 is minor Hb after birth and consists of two α and two δ chains. The most common Hb variants worldwide in descending order of prevalence are HbS, HbE, HbC and HbD. All of these hemoglobin's have single amino acid substitutions in the β chain. Normal adult hemoglobin consists primarily of hemoglobin's A (90-95%), A2 (2-3%), F (0.5%), A_{1a} (1.6%), A_{1b} (0.8%), and A_{1c} (3-6%). Glycosylated hemoglobin's (GHb) are the minor hemoglobin molecules separable by chromatographic techniques into three major components: A_{1a}, A_{1b}, and A_{1c}. Hemoglobin A_1 refers to a combination of these three components [83].

Important perspective studies on chronic complications of Diabetes mellitus allowed us to establish with absolute certainty the role of glycosylated hemoglobin (HbA_{1c}) as a marker of evaluation of long term glycemic control in diabetic patients and the strict relationship between the risk for chronic complications and HbA_{1c} levels. Diabetes Control and Complication Trial (DCCT), a great extent study, has demonstrated that the 10% stable reduction in HbA_{1c} determines a 35% risk reduction for retinopathy, a 25- 44% risk reduction for nephropathy and a 30% risk reduction for neuropathy [84].

4.1. Glycosylation process

Glycosylation is a non-enzymatic reaction between free aldehyde group of glucose and free amino groups of proteins. A labile aldiminic adduct (Schiff base) forms at first, then, through a molecular rearrangement, a stable ketoaminic product slowly accumulates.

In the hemoglobin, the preferential glycosylation site is the amino-terminal valine of the β chain of the globin (about 60% of glycosylated globin). Other sites are: lysin 66 and 17 of the β chain, valine 1 of the α chain. The term HbA_{1c} refers to the hemoglobin fraction of the glucose bound stably (ketoamine) to beta terminal of valines.

4.2. Other proteins which undergo glycosylation

Albumin, α_2 macroglobulin, antithrombin III, fibrinogen, ferritin, HDL, LDL, transferrin; all of them are short half-life proteins. The glycosylation process of short half-life proteins stops at the formation of the stable ketoamine adduct.

4.3. Advanced Glycosylation End products (AGE)

The long half-life proteins such as actin, collagen, fibronectin, myelin, nucleoproteins, spectrin, and tubulin can also be glycosylated. These long half-life proteins (myelin and collagen) undergo a complex and irreversible rearrangement process, with the formation of Advanced Glycosylation End products (AGE). AGE form a family with many compounds, only partially identified; they accumulate in the structural proteins modifying the function of them. They bind to specific macrophage receptors inducing a release of hydrolytic enzymes, cytokines and growth factors able to promote the synthesis of fundamental substance and, acting at intracellular level, to determine a damage of the nucleic acids [85, 86].

Three mechanisms have been postulated that explain how hyperglycemia causes tissue damage: nonenzymatic glycosylation that generates advanced glycosylation end products, activation of PKC, and acceleration of the aldose reductase pathway. Oxidative stress seems to be a common to all three pathways.

5. Note on laboratory aspects

5.1. Microalbuminuria estimation

5.1.1. Sample handling

The collection of sample is very important when you are measuring MA. As many factors will alter the value and errors may occur due to improper aseptic precautions, improper storage and handling. After the collection it is preferable to measure on the same day and if urine albumin is not estimated immediately then urine can be stored at 4°C. Alternatively, 2ml of 50 g /L sodium azide can be added per 500ml of urine. Specimens are stable for at least 2 weeks at 4°C and 5 months at -70°C. Freezing samples may decrease albumin but mixing immediately before assay eliminates this effect [87].

Albumin excretion varies with physiological factors like exercise posture, diuresis. Thus samples should not be collected after exercise, in the presence of urinary tract infection, during acute illness, immediately after surgery or after an acute fluid overload.

The following are considered acceptable [88]:

- 24 hour collection is preferred by some centers but this is cumbersome and errors may occur due to improper sample collection and transport.
- Overnight (8 - 12 hour) urine sample collection
- Short term urine collection i.e. 1-2 hour collection (in laboratory or clinic)

- Early morning mid-stream urine sample is usually rather concentrated and using this sample has good correlation between the excretion rate and concentration of albumin.

Many conditions can give a false positive value. Some of these common conditions are shown in table 6:

• Acute hyperglycemia	• Urinary tract infection
• Hypertension- Independently causes microalbuminuria	• Cardiovascular diseases- Independent of diabetes
• Heavy exercise- Due to increased protein catabolism and altered renal circulation	• Febrile condition and Stress
• Contamination with seminal or menstrual fluid- Which has more amount of albumin	

Table 6. Various factors affecting microalbumin estimation

Semi quantitative methods	
	Principle
Micral microalbumin urine test strip	Immunochemical strip test is specific for albumin. Albumin in the sample is bound by soluble conjugate of antibodies and the β-galactosidase enzyme marker. Conjugate-albumin complexes are separated and the β -galactosidase enzyme reacts with a substrate to produce a red dye. The intensity of the color produced is proportional to the albumin concentration in the urine.
Clinitec Microalbumin	The test strip is based on dye binding by albumin method. It uses the high affinity dye bis (3,3′-diiodo- 4, 4′-dih ydroxy-5, 5′-di nitrophenyl)-3,4,5,6-tetrabromosulfonephthalein. At a constant pH, the strip turns blue in the presence of albumin, and color is directly related to albumin concentration in the urine sample.
Quantitative	
Immunoturbidimetry	In this process turbidity is produced by an immune complex reaction. This causes a reduction in the intensity of light as it passes through the solution. Turbidimetry is the measurement of this loss in intensity because of scattering, absorption or reflection of the incident light in the angle/direction of the incident light.

	Most colorimeters and spectrophotometers can measure turbidity with good precision and accuracy. This is the most widely used test as it can be done on most semi auto chemistry analyzers. It can even be done on automated chemistry analyzers.
Nephelometry	This assay is also based on scatter detection but unlike turbidimetry it measures scattered light at 90° to the incident light. The instrument is called a nephelometer. It is more sensitive than turbidimetry.
Radio immunoassay (RIA)	This assay procedure involves competitive binding between radio labelled and unlabelled molecules of antigen to high affinity, specific antibody. The amount of unlabelled antigen present in the specimen is measured by its competitive effect on the labelled antigen for limited antibody sites. It involves the use of radio isotopes like tritium (^{3}H), ^{131}I or ^{125}I as labels. It has high sensitivity and specificity. The sample values are determined by comparison with a calibration curve. The advantages are sensitivity and precision, whereas the disadvantage is short shelf life and radioactivity of the reagents.
Chemiluminescent immunoassay (CLIA)	Chemiluminescence is a chemical reaction that emits energy in the form of light. When used with immunoassay technology, the light produced by the reaction indicates the amount of analyte in a sample. This again is of two types: Luminescent Immunoassay (LIA): Here the labelled and unlabelled antigen competes for the limited binding sites on the labelled antibody. An inverse relationship exists between concentration of labelled antibody bound to the antigen and the unlabelled antigen. Immuno Chemiluminometric assay (ICMA): This is a sandwich assay in which unlabelled antigen is sandwiched between antibody bound to paramagnetic particles and antibody labelled Acridinium ester (AE). A direct relationship exists between the concentration of antigen in the patient sample and the amount of light emitted during oxidation of the AE.

Note: Advantages of both RIA and CLIA are highly sensitivity, specificity and reproducible.
Disadvantages are unavailability, cost factor; proper infrastructure needed, radioactive hazards, Government permission for use of radioactive materials is the limiting factors.

Table 7. Methods of estimation [87, 88]

Marker	Type 1 diabetes sample assayed	Type 2 diabetes sample assayed
Glomerular transferrin	Urine	Urine
Fibronectin	Plasma	Urine
Serum laminin P1	-	Serum
Urine laminin P1	Urine	Urine
Type 4 collagen	-	Serum and urine
Heparan sulfate proteoglycan	Urine	-
Tubular proteins beta-2 microglobulin	Urine	Urine and blood
Retinol-binding protein	Urine and serum	Urine
Tamm-Horsfall protein	Urine	-
Alpha1-microglobulin	Urine	Urine
N-acetyl-beta-D-glucoseaminidase	Urine and serum	Urine and serum
Cholinesterase	-	Urine
Gamma-glutamyl transpeptidase	-	Urine
Alanine aminopeptidase	-	Urine
Tubular antigens,brush-border antigen	-	Urine

Table 8. Newer markers of diabetic nephropathy [89]:

6. Methods of estimation of glycated hemoglobin

In the last 20 years improved techniques in laboratory and new electrophoretical, chromatographic and immunological methods available, gave us a greater reliability on results. However the use of different methods, the lack of a common calibration concerning the same method and the variability of instrumentation do not make reproducible results yet in different laboratories. For this reason studies and procedures of standardization are going on [89]. Methods of GHb assays have primarily evolved around three basic methodologies:

1. Based on difference in ionic charge.
2. Based on structural characteristics.
3. Based on chemical reactivity.

The main methods are,

- Cation exchange chromatography
- Affinity chromatography
- High performance liquid chromatography
- Isoelectric focusing
- Radioimmunoassay
- Spectrophotometric assay
- Electrophoresis/Electroendosmosis
- Electrospray mass spectrometry

6.1. Specimen collection

Handling of specimens before the assay is important as short period of hyperglycemia before blood is taken, leads to an acute increase in the formation of aldimine which may increase the concentration of glycosylated hemoglobin by 10-20%--for example, from 9% to 11% of total hemoglobin-thus reducing the reliability of the test as a measure of long term diabetic control.

Blood samples should therefore be treated to remove the aldimine residues before the assay [91]. In measurement of HbA_{1c} the prevalence of the most common hemoglobin variants (HbS, HbC, and HbD) depends on the genetic background of the population being analysed. There are many Hb variants that result in false low HbA_{1c} level in diabetes. More than 700 Hb variants are known and about half of these variants are clinically silent, their presence may falsely interfere with the measurement of HbA_{1c} by HPLC. Hence, the identification of Hb variants is important to avoid inaccurate HbA_{1c} results [92].

Recent reports have shown that the concentration of total glycosylated hemoglobin measured by commonly used methods may change significantly over a period of hours. This reflects the short term fluctuations in glucose concentration. It is now realized that these rapid changes will depend on the synthesis or dissociation of the labile fraction of HbA_{1c}, which is not separable from the stable form of HbA_{1c}, by most routine methods. In most cases, the labile fraction constitutes approximately 10% of the total glycosylated hemoglobin. This may increase to 25% when plasma glucose concentrations are high, as in poor glycemic control.

These day to day variations in glycosylated hemoglobin concentration secondary to changes in serum glucose are negligible in stable diabetics, but are very wide in unstable diabetics and are almost entirely dependent on the prevailing plasma glucose concentration. Thus, during poor glycemic control there will be large swings in plasma glucose levels, a single HbA_{1c} measurement may be misleading as an index of long term control. It would therefore make sense to measure the stable fraction of glycosylated hemoglobin. However, this is not routinely available because most laboratories measure total HbA_1 or HbA_{1c}, which includes both labile and stable components. Therefore, in unstable diabetics, HbA_1 measurements should be interpreted in relation to the simultaneous glucose concentration. To minimize the contribution of the labile fraction, glycosylated hemoglobin should be measured when the plasma glucose concentration is within or near the normal range.

Physicians should be aware of the expected variation in HbA_{1c} during conditions such as [93],

- False increases in HbA_{1c} levels may occur in the presence of HbF (Ex: Hereditary persistence of fetal Hb) and other negatively charged hemoglobin's
- HbA_{1c} levels may also be increased in patients with renal insufficiency, caused by hemoglobin carbamylation resulting from condensation of urea with the same site to which glucose attaches.
- Increased HbA_{1c} occurs with advanced malignancy and iron deficiency anemia.
- Increased levels can be seen in people with a longer red blood cell lifespan, such as with Vitamin B_{12} or folate deficiency.
- Splenectomy can result in elevated levels of glycosylated hemoglobin

- False decreases may result when HbS (Ex: Sickle cell disease) or other positively charged variants are present
- Hemolytic anemia and chronic blood loss result in decreased red cell life span and therefore lower glycosylated hemoglobin levels.

6.2. Factors and clinical conditions affecting glycated hemoglobin levels are shown below [78]

Increased glycated hemoglobin	Decreased glycated hemoglobin
• Iron and Vitamin B12 deficiency • Alcoholism • Chronic renal failure decreased intraerythrocyte pH • Increased erythrocyte life span: Splenectomy • Hyperbilirubinemias • Large doses of aspirin • Chronic opiate use	• Administration of erythropoietin, Iron, Vitamin B12, • Reticulocytosis, Chronic liver disease. • Certain hemoglobinopathies, increased intra-erythrocyte pH. • Decreased erythrocyte life span: hemoglobinopathies, splenomegaly, rheumatoid arthritis and drugs such as anti-retrovirals, ribavirin, dapsone therapy. • Hypertriglyceridemia.

Table 9.

6.3. Advantages and disadvantages of various HbA1c assay methods

Method	Advantages	Disadvantages
Ion Exchange Chromatography	• Can inspect chromograms for Hb variants. • Measurements with great precision.	• Variable interference from hemoglobinopathies, HbF and carbamylated Hb but the current ion exchange assays correct for HbF and carbamylated Hb does not interfere.
Boronate Affinity	• Minimal interference from hemoglobinopathies, HbF and carbamylated Hb.	• Measures not only glycation of N-terminal valine on beta chain, but also beta chains glycated at other sites and glycated alpha chains.
Immunoassays	• Not affected by HbE, HbD or carbamylated Hb • Relatively easy to implement under many different formats.	• Affected by hemoglobinopathies with altered amino acids on binding sites.

Table 10.

7. Diagnosis of diabetes, its complications and management

Diabetes screening is recommended for:

- Overweight children
- Overweight adults (BMI greater than 30)
- Adults over age 45
- Family history of diabetes along associated risk factors such smoking, hypertension etc.

The diagnosis of diabetes is mainly done by using Oral Glucose Tolerance Test (OGT) and the values are shown in Table:

NEW CRITERIA FOR DIAGNOSING DIABETES IN ADULTS	NORMAL PLASMA GLUCOSE VALUES FOR ADULTS		
One or more of the following must be present: 1. Fasting plasma glucose level of > 126 mg/dL on at least two separate occasions. 2. Random plasma glucose level of > 200 mg/dL with signs and symptoms of diabetes. 3. Fasting plasma glucose level < 126 mg/dL but 2 hour glucose concentration of > 200 mg/dL during a 75-gram oral glucose tolerance test.	Fasting After 75g oral glucose load	Time Zero 30 min 60 min 90 min 120 min	< 115 mg/dL (6.4 mM) < 200 mg/dL (11.1 mM) < 200 mg/dL (11.1 mM) < 200 mg/dL (11.1 mM) < 140 mg/dL (7.8 mM)

Table 11.

7.1. Frequency of visits and laboratory testing

The recommended frequency of follow-up is 3-6 months for patients with type 1 diabetes and for type 2 diabetes patients depending on the glycemic status.

Every 3 - 6 months	**Yearly**
• Glycosylated hemoglobin • Electrolytes, BUN and creatinine • Physical examination including foot examination by filament testing (Carville approach)	• TSH • U/A or urine for microalbumin • Complete chemistry panel (lipids, • LFT, electrolytes, BUN & creatinine) • Ophthalmology examination • Podiatry and nutrition

Table 12.

The frequency of laboratory assessment is subject to flexibility, based on clinical judgment, patients' current control of diabetes, and past laboratory values.

Podiatry if any evidence of neuropathy or breakdown of skin integrity, and nutrition, if dietary non-compliance is suspected.

7.2. Microalbumin testing

Type 1: Annual screening for type 1 diabetes should begin at puberty and for those patients who have had the disease for 5 years.

Type 2: Initial testing at diagnosis and thereafter annual screening needed.

Note: Microalbuminuria is urinary albumin excretion between 30-300 mg per day without an alternative explanation (e.g. urinary tract infection, heart failure, exercise in past 48 hours and blood glucose > 200 mg/dL). If no protein is found in a urine analysis, then a 24 hour urine collection for microalbumin or a spot urine albumin-creatinine ratio may be used (abnormal if > 30 mg albumin/ g creatinine) for screening.

7.3. Retinopathy screening

Baseline Screening:

- For patients with type 1 diabetes who are 13 years of age or older and who have had the disease for 5 years, a baseline screening examination is recommended, and yearly thereafter.
- For patients with type 2 diabetes, a baseline screening examination is recommended at the time diagnosis and yearly thereafter.

Diabetic retinopathy is the leading cause of legal blindness among Americans, aged 20-74. It is highly correlated with patient age and duration of diabetes. Visual loss secondary to diabetic retinopathy is largely preventable if screening is universal and appropriate treatment follows screening.

7.4. Vaccines

- Pneumovaz every five years.
- Influenza vaccine annually.

Regular physical activity: Helps in movement of blood glucose into tissues.

7.5. ACE inhibitors

Recommendations:

- All patients who demonstrate microalbuminuria should be prescribed ACE inhibitors to slow the progression of nephropathy whether they are hypertensive or normotensive.
- Patients with type 1 who are hypertensive and do not demonstrate microalbuminuria should be prescribed ACE inhibitors. Such patients usually develop microalbuminuria in concert with hypertension and are best served by controlling blood pressure initially with ACE inhibitors.

- ACE inhibitors should not be used in pregnant women due to the risk of fetal morbidity and mortality.

NOTE:

ACE inhibitors should be titrated as high as the patient tolerates without orthostatic symptoms, hyperkalemia and /or increasing renal insufficiency.

7.6. Oral hypoglycemic drugs and/or insulin therapy needed

Some of the drugs used are,

- Sulphonylureas

First generation	Second generation
Tolbutamide	Glipizide
Tolazamide	Glyburide
Acetohexamide	Glibenclamide
Chlorpropamide	Glimepiride

Table 13.

- **Meglitinides:**Repaglinide, Nateglinide etc
- **Biguanides:** Metformin etc
- Thiazolidinediones: Pioglitazone etc
- **Alpha-glucosidase:** Acarbose, Miglitol etc

7.7. Life style changes such as cessation of smoking is necessary.
7.8. Health education programs should be started.
7.9. Self-monitoring of plasma glucose using glucometer.
7.10. Management of diabetic complications.

8. Conclusion

The most common chronic complication in diabetic patients is ESRD and several factors contribute to the development of renal damage such as genetic factors, hypertension and hyperglycemia.

The suspected cases of diabetic nephropathy will also invariably have diabetic retinopathy and more predisposed for cardiovascular events and mortality. That's why it's very important to prevent the condition then providing treatment. This is possible, as the progression of diabetic nephropathy is slow and can be detected at an early stage. Microalbuminuria is an early indicator of diabetic nephropathy and urine examination for micro albumin is routinely done to detect and monitor the progression of nephropathy. As many factors can interfere with the estimation of micro albumin, it is very important that high standards are maintained while estimating the MA levels. Other than microalbuminuria various newer markers have come, which still have to be studied for their probable role in its prevention of diabetic nephropathy.

Since, early detection of microalbuminuria can help in early diagnosis of diabetic nephropathy, adequate care and precautions has to be taken while estimating it.

9. Further research

Further studies have to be conducted to find better markers for chronic complications. Regular health education programs has to be conducted at an regular intervals, so that patients lead a better life.

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