EVALUATION OF METHOD FOR GLYCATED HEMOGLOBIN AND ITS CORRELATION WITH MICROALBUMINURIA AS EARLY MARKERS OF NEPHROPATHY IN TYPE II DM

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Submitted by

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CERTIFICATE

This is to certify that the present thesis entitled "**Evaluation of method for glycated hemoglobin and its correlation with microalbuminuria as early markers of nephropathy in type II DM"** is the outcome of the bonafide work carried out by **Miss *Khadija Umar Abdullahi* (Registration Number *11410513)*** herself under our guidance and the contents of her thesis did not form a basis of the award of any previous degree to her and to the best of our knowledge to anybody else also. The thesis has not been submitted by the candidate for any research degree in any other university.

The dissertation is fit for submission to the partial fulfillment of the conditions for the award of M.Sc. in Clinical Biochemistry. Further certified that the candidate bears a good moral character and nothing adverse has been found against her.

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I hereby declare that the work embodied in this internship report was carried out by me under the supervision of Ms Anshula Sharma (Internal supervisor), Lovely Professional University and Prof. Dr. Uma Arora (External supervisor), PIMS hospital Jalandhar. This work has not been submitted in part or in full to any other university for any degree.

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DEDICATION

I dedicate this work to my lovely parents, late Prof. Shehu Umar Abdullahi and Hajiya Asma'u Imam.

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Khadija Umar Abdullahi

ABBREVIATIONS

ACE- Angiotensin-Converting Enzyme

ACR- Albumin creatinine ratio

ADA-American Diabetes Association

AER- Albumin excretion rate

BMI- Body mass index

BP-Blood pressure

DCCT- Diabetes Control and Complications Trial

DM- Diabetes Mellitus

DW-Distilled water

EDTA-Ethylene Diamine Tetra acetic acid

ERFD-Early Renal function decline

ESIMS-Electrospray ionization mass spectrometry

FBS-Fasting blood sugar

GFR-Glomerular filtration rate

GOD-POD- Glucose oxidase peroxidase

HbA1c- Glycated hemoglobin

HPLC- High Performance Liquid chromatography

IDDM- Insulin Dependent Diabetes Mellitus

IgG-Immunoglobulin G

PEITT- Particle –enhanced immunoturbidimetric method

PIMS- Punjab Institute of Medical Sciences

RBS- Random blood sugar

RT- Ratio of test

RC- Ratio of control

SD- Standard deviation

STD- Standard

TINIA- Turbidimetric inhibition immunoassay

Q.C- Quality control

WHO- World Health Organization

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Abstract

Insulin resistance is characterized by a subnormal response to a given concentration of insulin and can be measured indirectly by a fasting insulin level. Diabetes is a metabolic disorder which is characterized by hyperglycemia. Diabetic nephropathy is the leading cause of end stage renal disease and mortality. The aim of the study was (i) to correlate between two methods of HbA1c estimation; the column chromatography and the immunoturbidimetric method and (ii) to evaluate microalbuminuria and glycated hemoglobin (HbA1c) as early risk markers of nephropathy in type II diabetes mellitus. The present study includes two parts; the first part consists of forty known diabetic patients in which the method comparison of HbA1c was studied. The second part of the study comprises of eighty-four known diabetic patients and thirty healthy control subjects in which the presence of HbA1c and microalbumin was estimated and correlated. Informed consent of all patients and healthy controls was taken. Venous blood was obtained for HbA1c & plasma glucose for the first part of the study. For the second part, venous blood was obtained for HbA1c, and blood glucose, while their morning urine sample was obtained for detection of microalbuminuria. Statistical analysis was done using SPSS version 24. All p-values <0.05 were considered statistically significant. The two HbA1c methods were highly correlated (r=0.905). However, the mean of immunoturbidimetric method is slightly lower (5.23%) when compared with column chromatographic method (5.69%). Random Blood Sugar (RBS), HbA1c and microalbumin were the highest in diabetic patients (240.4±43.7 mg%), (9.96±1.21%), and(108.5±36.2mg%) when compared with non-diabetic healthy control subjects (93.4±16.1mg%), (4.80±0.50%)and (8.1±3.4 mg%) respectively. Microalbuminuria and HbA1c had a significant correlation (r=0.626, p<0.01). Microalbuminuria also had a good correlation with duration of diabetes (0.764, p<0.01)**.**

Key words: Diabetes, HbA1c, nephropathy, microalbuminuria, column chromatography, immunoturbidimetry.

INTRODUCTION

1.1 INTRODUCTION

Diabetes is a common endocrine disorder, characterized by persistent hyperglycemia as a result of inadequate insulin and/or insulin resistance (1). Untreated hyperglycemia can cause a lot of complications like neuropathy, nephropathy and retinopathy. Insulin resistance is characterized by a subnormal response to a given concentration of insulin and can be measured indirectly by a fasting insulin level: the increased levels of insulin correspond to higher degrees of insulin resistance (2). Factors that lead to insulin resistance include obesity, hypertension, high triglyceride, ageing, and a sedentary lifestyle (3). Diabetes mellitus (DM) is a major cause of chronic kidney disease (CKD) leading eventually to development of end stage renal disease (1).

The prevalence of diabetes is continuously increasing and the morbidity and mortality due to the disease is emerging as a major healthcare problem worldwide (4). A total of 347 million people had diabetes in 2004, of which the mortality was 3.4 million (5). The mortality was found to be more in low and middle income groups (6). This metabolic disorder in the USA, affects over 16 million people (6% of the population) and it is found that over 150,000 patients die from the disease and its complications yearly (7). WHO estimated that by the end of the year 2030, DM will be the seventh leading cause of death worldwide (8). Out of the two types of DM (i.e type I and II), type II is more prevalent (9). In most cases, patients with type II DM often have a long asymptomatic period of hyperglycemia and at the time of diagnosis, complications are seen. (10).

Metabolic disorders accompanied with diabetes result in pathophysiological changes due to hyperglycemia in various systems in the body (11). Because the complications of diabetes mellitus are related to glycemic control, hence normoglycemia is an appropriate goal for most of the patients. Assessment of HbA1c is a gold standard to check long-term glycemia in diabetic patients (12).

Glycated hemoglobin (HbA1c) is the presence of carbohydrate-protein linkage on the N-terminus of the β-chains of hemoglobin, predominantly HbA in adults (13). The percentage of glycated hemoglobin in the plasma of a patient provides an estimate of blood glucose levels over a period of 120 days (14).

The results of Diabetes Control and Complications Trial (DCCT) provide strong evidence that glycemic control as assessed by HbA1c testing predicts risk for developing diabetic complications in future. Control of HbA1c will help reducing the risk of complication (15,16).

Diabetic nephropathy is a long term complication of (diabetes mellitus) DM and is the chief cause of morbidity and premature mortality. The pathophysiologic basis for elevated urinary albumin excretion entails the binding of glucose to proteins resulting in excessive protein glycosylation with the buildup of advanced glycated end products. This leads to the deposition of advanced glycated end product on the glomerulus resulting in renal and glomerular hypertrophy, mesangial matrix accumulation and thickening of glomerular basement membrane. This abnormality permits the leakage of low molecular weight proteins like albumin (17).

Microalbuminuria is defined as Urinary albumin excretion between 30-300mg/day. In the early stages of the disease, there is an increase in urinary albumin excretion, which progresses to overt albuminuria and then to renal failure (18).

The early stage of diabetic nephropathy is considered to be microalbuminuria. Both among diabetic and non- diabetic subjects, microalbuminuria is also considered to be a predictor for cardiovascular disease and is one of the components of metabolic syndrome (19).

Screening for microalbuminuria and timely therapeutic intervention has become the standard of care worldwide as it can be reversed and the future development of overt diabetic nephropathy reduced (20).

In type II diabetic patients, the duration of diabetes and elevated glycemic control (HbA1c) well, predicted increased microalbumin excretion rate. As determination of microalbumin level in urine is an easy method of screening the diabetic patients, it may be useful to prevent the onset of future renal disease. A good glycemic control in the early stages reduces chances of microalbuminuria (21).

Several studies have shown a positive correlation between microalbuminuria and HbA1c. This study was carried out to compare the two methods for HbA1c estimation and also to correlate it with microalbuminuria and duration of diabetes. Microalbuminuria and HbA1c were studied as risk markers of renal damage.

REVIEW OF LITERATURE

2.1 REVIEW OF LITERATURE

Several studies have compared different methods of glycated hemoglobin determination. Also many studies have correlated HbA1c and microalbumin as markers of nephropathy in diabetic patients.

It has been found that type II diabetes mellitus has a strong genetic component and is found more frequently in certain families and ethnic minority groups, such as Hispanics, African Americans, Pacific islanders and American Indians (22).

Various methods exist to measure glycated hemoglobin with different values, making the comparison of the methods very difficult (23). Also factors such as types of anemia, pregnancy, splenectomy, transfusion and intake of medications (salicylates) influence many methods of glycated hemoglobin estimation (24).

Laboratories perform HbA1c assays according to the manufacturer's instructions with the commercially available methods which include affinity chromatographic methods, HPLC methods, atmospheric pressure ion exchange chromatography with disposable columns, electrophoretic methods, immunochemical methods (25).

A major drawback that limits clinical utility of HbA1c at present is the variation of HbA1c results when determined by different methods and by different laboratories (26). Lack of standardization is the major explanation for this phenomena and solving this problem has become a major priority of the National Diabetes Data Group in the US (27).

Numerous studies have compared different methods of HbA1c estimations. Therefore, with regard to this, the present study was undertaken to compare two routine methods; column chromatography and immunoturbidimetric method.

2.1.1 Comparison between different methods for HbA1c

Pecararo *et. al* (1979) compared the determination of HbA1c using colorimetric assay and ion-exchange chromatography.The two methods were highly correlated (r= 0.943). However, a unique advantage of the colorimetric procedure was the capability to estimate

HbA1c levels when variant hemoglobins, including fetal and sickle hemoglobins are present (28).

Klenk *et. al* (1982) compared the estimation of HbA1c by affinity chromatography with colorimetric and ion-exchange methods. Results showed that there was a correlation of 0.98 between ion-exchange method and affinity chromatography and colorimetric assay method. It was concluded that the affinity method offers a rapid, simple, precise and accurate alternative to methods currently in use and gives substantial freedom from many common interferences (23).

Latex-enhanced method on Dade dimension analyzer was compared with HPLC procedure in a study by Holownia *et. al* (1997). It was concluded that adaptation of the method for use with the Dimension analyzer is a valid method for quantifying % HbA1c (29).

Vucic *et. al* (1998) compared immunoturbidimetric assay with ion-exchange chromatography. Results showed that the immunoturbidimetric procedure produced results that were highly comparable with ion-exchange chromatography in a wide range of HbA1c values (30).

Electrospray ionization mass spectrometry (ESIMS) and a routine HPLC based ion-exchange procedure were compared to determine HbA1c in a study by Roberts *et. al* (2001). Results showed that ESIMS provides a precise measurement of HbA1c. The method is robust and could be proposed as a procedure to substantiate HbA1c measurement and/or calibration (31).

HPLC system, Tinaquant immune turbidimetric method and Bio Rad variant chromatographic method were compared in a study by Antunes *et. al* (2009). The results showed that HPLC Mono S is a precise, low-cost method which yields similar values to the Bio-Rad variant method on conventional HPLC equipment (32).

A study by Sahu *et. al* (2010) compared and correlated modified NBT reduction method with DCA + 2000 analyzer for the estimation of HbA1c. The study concluded that HbA1c testing by modified NBT reduction method was same as estimated by DCT 2000+analyzer. Hence method could have been used for routine monitoring of glycemic control level in diabetic subjects (33).

Moiz *et. al* (2012) compared an affinity chromatography micro column assay for glycated hemoglobin with electrophoretic and micro column ion exchange methods. Non-linear relationships between affinity methods on one hand and electrophoretic as well as ion exchange methods on the other hand indicated that affinity method measured not only β-terminal glycated hemoglobin but all glycated hemoglobins, providing enhanced sensitivity and diagnostic value (34).

Three methods were compared for the measurement of blood HbA1c in a study by Klenk *et. al* (1982). The results showed that there was good concordance between results of particle-enhanced immunoturbidimetric assay (PEITT) and HPLC method (r= 0.94). The average HbA1c measured by HPLC was higher than the other methods PEITT and TINIA. It was concluded that the PEITT method, which is reliable, faster and easier to perform, can be used as an alternative to TINIA and HPLC (35).

Sudhakar *et. al* (2014) compared three methods for measurement of blood HbA1c as to reliability, ease and time consumption. The methods included turbidimetric inhibition immunoassay (TINIA); which also required measurement of total hemoglobin, a particle-enhanced immune turbidimetric method (PEITT) without measurement of total hemoglobin, and high performance liquid chromatography (HPLC). It was found that the PEITT method, was reliable, faster and easier to perform, and can be used as an alternative to TINIA and HPLC measuring system within known precision limits (36).

Karami *et. al* (2014) compared three methods with HPLC method. Boronate affinity binding (Nycocard), enzymatic (Diazyme) & column chromatography (Biosystem), with HPLC in order to declare which method gave consistent values and correlated with those of HPLC so as to replace that in clinical laboratories. Results showed that values obtained by the Diazyme device were closer to HPLC compared with the other two methods (37).

Two methods, immunoturbidimetry and liquid chromatography method were compared by Abdallah (2015). The results of the study showed that turbidimetry was a useful method for determining glycemic levels above 200mg/dl (38).

Affret (2015) compared the performance of A1cNow self-check device with laboratory TOSOH analysis. The findings showed that there was good correlation between the two methods. Regression analysis showed $r_2 = 0.93$ for both methods. Lay users found the A1cNow self-check easy to use, and both lay users and health care professionals were able to measure HbA1c accurately (39).

2.1.2 Role of Microalbumin as marker for Diabetic Nephropathy----

Diabetic nephropathy is one of the leading causes of end stage renal disease in the world (40).

The relation between blood pressure and urinary albumin excretion in the development of microalbuminuria were examined in a study by Elisabeth *et. al* (1990). The results showed that significant elevation in urinary albumin excretion precedes the increase of systemic blood pressure during the development of nephropathy in insulin-dependent diabetes mellitus (41).

Mogensen (1990) in a review stated that the development of severe kidney impairment was seen in large percentage of patients with microalbuminuria. Parenteral predisposition to hypertension is not seen in all studies and therefore may not be a decisive factor, and it cannot be used in prediction of nephropathy. Prediabetic blood pressure may predict nephropathy in certain type II diabetic patients, but elevated blood pressure seems to develop after early microalbuminuria and is likely to be an aggravating factor in established microalbuminuria in type I diabetic patients (42).

It was found out that the rate of increase in albumin excretion rate (AER) correlated with mean HbA1c in type I and type II diabetic patients in a study by Gilbert *et. al* (1993). The study concluded that long-term glycemic control correlated with the rate of development of early renal abnormalities (43).

The relationship between HbA1c and microalbuminuria in diabetic patients (type 1) was examined in a study by Krolewski *et. al* (2016). Results showed that the prevalence of microalbuminuria was 18% in the patients. It increased with the duration of diabetes, and ased

non-linearly with the HbA1c value. The risk of microalbuminuria in diabetic patients increased abruptly with HbA1c value of 8.1%, suggesting that efforts to reduce the frequency of diabetic nephropathy should be focused on reducing HbA1c values (44).

Huraib *et. al* (1995) in a study found that there was a significant correlation between the presence of hypertension, duration of diabetes and development of diabetic nephropathy. Hypertension and duration of diabetes mellitus are important risk factors in the development of diabetic nephropathy (45).

The prevalence and risk factors for microalbuminuria among south Indian type II diabetic patients was determined in a study by Varghese *et. al* (2001). The result of the study found that the prevalence of microalbuminuria was 36.2%, and also the prevalence increased with increase in duration of diabetes. The study also showed that regression analysis revealed age, diastolic blood pressure, HbA1c, Fasting Blood Sugar (FBG) and duration of diabetes to be associated with microalbuminuria (46).

The prevalence of microalbuminuria among type II diabetic patients was studied by Parving *et. al* (2006). The overall global prevalence of normo, micro-, and macroalbuminuria was 51%, 39% and 10% respectively. The Asian and Hispanic patients had the highest prevalence of a raised urinary albumin /creatinine ratio (55%) and Caucasians the lowest (40.6%) (47).

A study by Koroshi (2007) showed that microalbuminuria as an earlier sign of vascular damage. It is a marker of general vascular dysfunction and nowadays is considered a predictor of worse outcomes for both kidney and heart patients. Cardiovascular and renal risk is elevated even in the high normal range of microalbuminuria (48).

Perkins *et. al* (2007) in a study found that the risk for early renal function decline depended on whether microalbuminuria regressed, remained stable or progressed. The risk for decline was higher after age 35 years or when glycated hemoglobin exceeded 9%, but did not vary with diabetes duration, smoking, BP or angiotensin converting enzyme inhibitor treatment. Progressive renal function decline in type I diabetes is an early event that occurs in a large proportion of patients with microalbuminuria (49).

The prevalence of microalbuminuria in relation to duration of diabetes, BMI, serum creatinine and HbA1c in an ethnic group of type II diabetes mellitus subjects, residing in Karachi was determined in a study by Sheikh *et. al* (2009). The result of the study showed that microalbuminuria had a highly significant correlation with duration of diabetes, serum creatinine ($p<0.01$), HbA1c ($p<0.05$), BMI ($p<0.024$). A strong correlation was found between age and serum creatinine. It concluded that early onset of microalbuminuria in the selected community h could be due to poor glycemic control (High HbA1c >7%) or heredity factors (50).

Perkins *et. al* (2009) in a review stated that in type I diabetes mellitus, regardless of the course of albumin excretion, a subset of approximately third individuals will initiate a process of Early Renal function Decline (ERFD) to advanced stages of chronic kidney disease. The existence of pathogenic mechanisms for the two independent early phenotype-microalbuminuria and ERFD are further supported by the findings of recent clinical trials that imply that, therapy successfully controlling urinary albumin excretion may not have impact on ERFD (51).

Naveen *et. al* (2012) identified that the risk of microalbuminuria increased with poor glycemic control and persistent increase in glycated hemoglobin and microalbuminuria may be considered as risk markers in diabetic nephropathy (52).

In another study by Maiti *et. al* (2012), the study aimed to examine the relation between microalbuminuria, HbA1c as well as duration of diabetes. Result of the study showed that there was a positive correlation found between BMI and microalbuminuria. However, there was no correlation between microalbumin and age. There was a positive correlation between microalbumin and duration of diabetes as well as microalbumin and HbA1c (53).

A study by Khan P *et. al* (2012), aimed at examining the relationship of glycemic control with prevalence of microalbuminuria in diabetic patients. The study found that the prevalence of microalbuminuria was 29.5%. The study concluded that uncontrolled diabetes is strongly associated with prevalence of microalbuminuria, and that screening for microalbuminuria and HbA1c should be done in all type II diabetic patients (54).

Chae *et. al* (2012) in a study evaluated the clinical values of spot urinary albumin :creatinine ratio and serum cystatin C for the assessment of diabetic nephropathy instead of 24hr urine

microalbumin in children and adolescents with diabetes. The study evaluated the validity of spot urine albumin: creatinine ratio and serum cystatin C, and then compared them to 24 hr urine microalbumin and creatinine clearance. Results showed that spot urine albumin to creatinine ratio correlated with 24 hr urine albumin excretion and creatinine clearance. The study concluded that both the measurements of spot urine albumin:creatinine ratio and serum cystatin C might better predict the presence of diabetic nephropathy (55).

Kundu *et. al* (2013) examined microalbuminuria and correlated levels to glycated hemoglobin level and duration of diabetes. Results showed that microalbumin levels were linearly correlated to the duration of diabetes and HbA1c. Study showed that impaired glycemic control is associated with significant elevations in urinary microalbumin levels. Furthermore, there is an increased urinary microalbumin level with increased duration of diabetes, which suggests that the detection of increased microalbumin level at the initial stage can reduce the clinical and economic burden of diabetic complications in future (56).

Kondaveeti *et. al* (2013) in a study found that microalbuminuria and serum creatinine were the highest in uncontrolled diabetes mellitus subjects compared with the controlled diabetes mellitus subjects respectively. Microalbuminuria had a significant correlation with the duration of diabetes showing that the risk of microalbuminuria increased with a poor glycemic control (57).

A study by Bakris *et. al* (2013) studied the long term complications among type II diabetics and their association with HbA1c. The result of the study showed that the high level of HbA1c predisposes patients to long-term complications (58).

Kondaveeti *et. al* (2013) in another study found that the risk of microalbuminuria increases with poor glycemic control. Persistent increase in glycated albumin and microalbuminuria may be considered as risk markers in diabetic nephropathy (59).

The relationship between glycated hemoglobin and diabetic nephropathy were correlated in a study by Kumar *et. al* (2014), which showed that the incidence of microalbuminuria increased with age and increased duration of diabetes mellitus. The study also found that there was a relationship between diabetic nephropathy and retinopathy with a significant correlation (60).

Mohammed *et. al* (2014) in a study has shown a significant correlation of glycosylated hemoglobin and urinary albumin in diabetes mellitus with vascular complications. Hence measurement of glycated hemoglobin along with microalbumin is significant as an early marker in predicting nephropathy in uncontrolled type II diabetes mellitus with complications (61).

A study by Popoola and Olotuah (2014) examined the association between fibrinogen, microalbumin and HbA1c. The result showed that the diabetic patients with microalbuminuria tend to have higher fibrinogen. Fibrinogen was found to be associated with microalbumin loss, BMI and HbA1c (62).

The relationship between microalbumin and HbA1c levels in diabetic patients was examined in a study by Anwarullah *et. al* (2014). The result of the study showed that microalbumin levels were linearly correlated to those of HbA1c levels (63).

In a study by Seedat (2006), urinary microalbumin, microalbumin creatinine ratio in random sample and microalbumin in the 24 hr sample was correlated with the various glycemic and hypertension markers and the study revealed that although all the three parameters had positive correlation with the markers of glycemia and hypertension, the urine microalbumin creatinine ratio of the random sample had a better positive correlation. Also HbA1c and, FBS had a better correlation with the microalbumin creatinine ratio (64).

The relationship between HbA1c and urinary microalbumin in patients of diabetes mellitus was studied by Tandon *et. al* (2015). Microalbuminuria showed a significant correlation with HbA1c and duration of diabetes, thus serving as an invaluable tool in monitoring of glycemic status and screening for diabetic nephropathy (65).

Acharya *et. al* 2015 examined the status of urinary microalbumin in relation to duration of diabetes and HbA1c levels in patients with type II diabetes mellitus. The result of the study suggested that the overall prevalence of microalbuminuria was 39.6%. Also microalbuminuria had a highly significant correlation with duration of diabetes ($r=0.471$ $p<0.05$). Also it suggested that there is a positive correlation between microalbuminuria with HbA1c levels, although statistically insignificant ($r=0.245$, $p>0.05$) (66).

The early studies of the relationships of increasing blood pressure, increasing albumin excretion and declining GFR published by Parking and others (2015) demonstrated the importance of blood pressure reduction in reducing the albumin excretion rates and attenuating the decline in GFR. Subsequently, modulating the RAAS (rennin-angiotensin-aldosterone system) and ACE-inhibitors (Angiotensin-converting enzyme) and ARBs has demonstrated favorable effects on measures of diabetic nephropathy independent of reducing the blood pressure (67).

Omar *et. al* (2015) in a study found that 88.8% of patients with poor glycemic control had microalbuminuria compared to 53.8% with accepted glycemic control. The difference was more statistically significant among the adolescent age group. Microalbuminuria was found in 77.2% of children with duration of type I diabetes of less than five years but the highest proportion was found when the disease duration was more than ten years. Also there was no significant difference in systolic and diastolic blood pressure among diabetic children with and without microalbuminuria (68).

A study by Aggarwal and Kumar (2016) showed that albumin:creatinine ratio (ACR) was found to be significantly elevated in both type I and type II diabetes mellitus and it increased in diabetics with poor glycemic control, longer duration of diabetes and smoking (69).

A study by Kumar and Prasad 2016 determined the evaluation of HbA1c and risk of microalbuminuria in patients with type I diabetes mellitus. The result of the study found that there was a positive correlation of microalbuminuria with duration of diabetes and level of glycemic control (measured by HbA1c levels). The result is found to be in accordance with many previous studies (70).

Mandal and Jyothrimayi 2016 aimed at comparing the levels of HbA1c and microalbumin in type II diabetic patients with complications and those subjects without complications. Result of the study showed that microalbumin level is at a significantly higher range with high HbA1c level in patients with complications as compared to those without complications (71).

2.2 AIM AND OBJECTIVES

-To correlate between two methods of HbA1c estimation : column chromatography and immunoturbidimetric method.

-To evaluate the role of HbA1c and microalbumin estimations as markers of nephropathy.

MATERIALS AND METHODS

3.1 MATERIALS AND METHODS

Forty patients who had been diagnosed with type II diabetes mellitus with duration of more than one year were included in the study. The patients were receiving treatment and counseling on monthly basis at the diabetic clinic in Punjab Institute of Medical Sciences, Jalandhar. Their fasting blood glucose was being measured once in a month while HbA1c was checked once every three months. Informed consent was taken from the patients before including them into the study. Another group consisting of eighty-four known diabetic patients (56 females and 28 males), admitted as inpatients and attending outpatient clinics of the hospital ,were also included in the present study. Thirty healthy subjects were included in the control group.

3.1.1 Inclusion criteria

Known diabetic patients, aged between 35 to 90 years, of either sex, were chosen for the study.

3.1.2 Exclusion criteria

Diabetic patients who had macroalbuminuria were excluded from the study. Patients with mental illness or suffering from serious medical conditions like jaundice, anemia etc, were excluded.

A structured questionnaire regarding the demographic data such as name, age, sex, duration of diabetes, presence or absence of hypertension, smoking habit was recorded from each patient.

The blood samples (1ml) were collected by the technicians at the Central Blood Collection Centre from patients, in EDTA containers for HbA1c and in oxalate and fluoride vials for glucose. The samples were sent to laboratory to measure HbA1c with column chromatography and immunoturbidimetric methods. The blood samples for HbA1c were kept in refrigerator (4^{o}C) until analyzed. Blood sample was analyzed for blood glucose using GOD-POD method. For microalbumin estimation, morning urine sample was taken. All urine samples were tested for the presence of albumin by pyrogallol red method.

3.2.1 Materials

- Syringes
- Test tubes
- Tourniquet
- Cotton swab
- Urine containers
- Centrifuge
- Test tube rack
- Fully automated analyzer (BS-400 chemistry analyzer)
- Semiautoanalyzer (ERBA CHEM-7)

3.2.2 BS 400 CHEMISTRY ANALYZER FULLY AUTOMATED (MINDRAY MACHINE)

Fig. 1: BS-400 Chemistry analyzer

Principle

Basic operation (BS-400)

- Checking power before on.
- Power on.
- Start the operation software.
- Set up the analyzer.
- Load reagents.
- Check reagent inventory.

Starting Analysis

- Programming calibrator.
- Programming control
- Programming routine sample.
- Programming start sample.
- Programming reagent blank.
- Adding/ deleting sample and tests.
- Re-running sample.
- Editing test results.
- Printing
- Finishing analysis.
- Power off.

Power on

- Turn on the water unit.
- Place the main power on.
- Place the power on
- Press the power button on the monitor of the operation unit.
- Press the power button of the printer.
- The operation software will start automatically.
- Log in with user name and password, setting up the analyzer.

Before requesting the test, you must do the following

- Select test parameter:-

  Parameter----test-----basics
- Regents' setup: select reagents-------reagents' setup.
- Calibration: select parameter------- test------calibration, and set up calibrator and calibration.

Preparing reagents

- Reagent bottles are then loaded to their assigned positions on the reagents disk and then open.

Check reagents inventory

- When reagent pack is installed, the reagent inventory should be checked to ascertain the level of all reagents.

Start analysis

- When the system is in idle state, programming operation may proceed.

Programming calibrators

- If you are using the machine for the first time, calibration must be run.

Programming control

- Two quality controls are run daily for each test to check if the system is working normally or steadily.

Programming routine sample

- Request and run sample.
- Select samples, then sample request. The sample with desired test is then selected.
- After requesting, the sample is then load to their assigned positions on the sample disk.
- Start button is the click to start running the samples.

Technical specificity

- 400 test per hour.
- Analysis principle: colorimetry, turbidimetry, ISE method
- Reaction type: end point, fixed-time and kinetic.
- Sample disk :90 position
- Sample volume: 02-45 ul.
- Reagent disk: 80 position

- Reaction cuvette: 90 cuvette, 5mm 5mm 30mm optical path 5mm capacity 750 ®µl.
- Reaction temp: - 37C.
- Photometric system: static fibre optics and reversed optics of holographic concave flat- field gratings.
- Light source 12V, tungsten-halogen lamp, 20W.
- Wavelength: 12 W length (example):- 340nm,380nm,412nm,450nm,505nm,546nm,570nm,605nm,660nm,700nm,740nm,and 800nm.
- Measuring period: 9 Seconds.
- Deionised water consumption: 20 L/H.

3.2.3 SEMIAUTOANALYZER (ERBA CHEM -7)

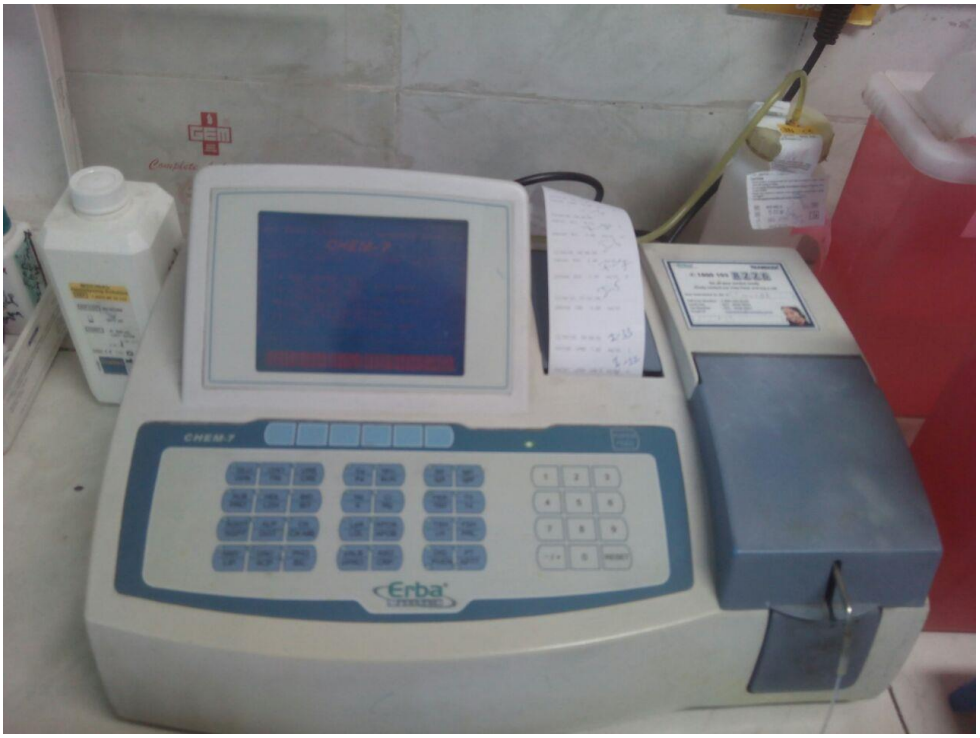


Fig. 2: Semiautoanalyzer

Standard operating procedure for CHEM-7 (semiautoanalyzer)

- The machine is switched on
- Distilled water is then aspirated in order to clean the tubings
- From the display key button, select the parameter
- Distilled water is then aspirated.
- Reagent blank is then aspirated
- The optical density of the blank is then saved
- Standard is then aspirated
- The machine will measure the concentration of the standard
- The test is then run by pressing the aspiration button
- The concentration of the parameter will be calculated automatically and printed

- Rinse the machine with distilled water
- Switch off the machine (Note: Before aspirating the sample, reagent and water make sure that the probe is properly placed at the bottom of the container to avoid aspiration of air bubbles).

3.3 Estimation of HbA1c

Column chromatographic method (Ion-exchange resin method)

Ion exchange resin method employs the use of a kit containing chromatographic columns accompanied with ion exchange resin which should be used at room temperature. Its function is based on principle of ion exchange. According to the method, a separate ion exchange column was used for each sample and finally, the supernatant was collected for HbA1c estimation.

For the collection of supernatant, whole blood (100µl) and control (100 µl) was mixed with 500µl of lyzing reagent to obtain hemolysate. The hemolysate was then incubated for a period of five minutes at 37^{o}C. 100µl of the hemolysate was then added into the column containing ion exchange resin which was then rotated for five minutes. Then allowed to stand for ten minutes and then supernatant was separated using resin separator. The absorbance of the supernatant was then measured on spectrophotometer at wavelength of 415nm to get the total glycated hemoglobin value.

10µl of the hemolysate was also added to 2.5ml distilled water to the test and control tubes. The absorbance of these tubes was measured at 415nm to obtain the total hemoglobin values. HbA1c was calculated using the following formula:

$$\text{Glycated Hb in \%} = \frac{\text{Ratio of Test } (R_T)}{\text{Ratio of control } (R_C)} \times 10$$

10 is the concentration of the control provided in the kit

$$\text{Ratio of Test } (R_T) = \frac{\text{Absorbance of test Glycated Hb}}{\text{Absorbance of test Total Hb}}$$

$$\text{Ratio of Control } (R_C) = \frac{\text{Absorbance of control Glycated Hb}}{\text{Absorbance control of Total Hb}}$$

Reagent stability

Contents are stable at 2-8^{o}C till the expiry mentioned on the label.

Linearity

The Glycated Hb procedure shows linearity for Glycated Hb levels in the range of 4 – 20% (72).

This is a very time consuming (45 minutes) and should be administered very carefully.

3.3.1 Immunoturbidimetric method (BS 400 Chemistry analyzer)

Hemolysates were prepared by mixing 1000µl of hemolyzing reagent with 20µl of whole blood and keep it at room temperature for approximately 5 minutes or until lysis was completed in accordance with the testing method. The HbA1c values of the blood samples were measured directly without measurement of Total Hb. HbA1c and Total Hb in hemolyzed sample were bound with equal affinity to solid-phase particles in reagent. Subsequently mouse antihuman HbA1c monoclonal antibody was added to attach to particle bound HbA1c. Goat antimouse IgG polyclonal antibody interacts with the monoclonal mouse anti-human HbA1c antibody to produce the agglutination reaction. Finally absorbance, which is proportional to the HbA1c bound to particles was measured at wavelength of 430nm. (73)

The result is reported in percentage and working with this instrument is very convenient compared to column chromatographic method requiring much less time.

3.3.2 Glucose estimation (GOD-POD method)

10µl of serum is added to 1000µl of reagent. The solution is incubated for 10 minutes. The absorbance is read at 540nm on a spectrophotometer. Glucose concentration is calculated as follows:

$$\frac{\text{O.D of Test}}{\text{O.D of standard}} \times \text{Concentration of standard}$$

Concentration of standard = 100mg%

Normal range for RBS = 70-140mg% (74)

3.3.3. Microalbumin estimation (Pyrogallol red method)

5µl of urine sample is added into 500µl of reagent. Absorbance is read at 600nm on a semiautoanalyzer.

Concentration of microalbumin in mg/dl is calculated as follows:

$$\frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times 1000$$

Normal range: 30- 300mg/dl (75)

RESULT

4.1 RESULT

A total of 40 samples were analyzed for HbA1c estimation. The mean age of the patients included in the study was 41.48±11.4years (range 25-48years). There were 32 (80%) females and 8 (20%) males. A comparison was made between two methods available in the laboratory for the estimation of HbA1c.

All statistics in the study was performed using SPSS statistical software version 24. The descriptive statistics for both the techniques are shown in table1. The mean HbA1c was slightly lower for immunoturbidimetric (5.23%) method than column chromatography (5.69%). The correlation analysis was also done between the results obtained by column chromatography and immunoturbidimetric method. The correlation coefficient was found to be 0.905. The result showed a good correlation between both methods. Column chromatographic method and RBS had correlation coefficient of 0.70 and immunoturbidimetric method and RBS had 0.78.

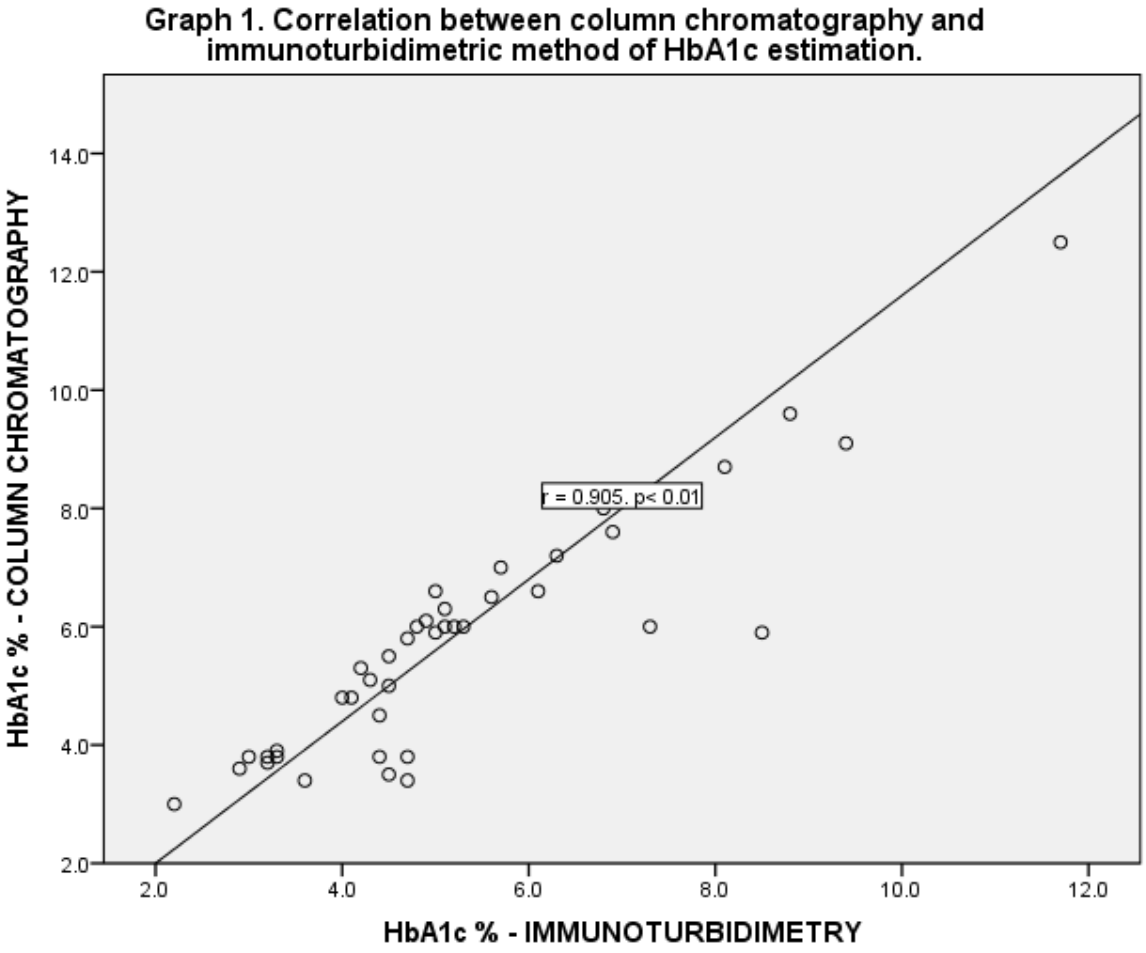
Table1. Values obtained by column chromatography and immunoturbidimetric method.

HbA1c (%)	Mean ± SD
Column chromatography	5.69 ± 1.99
Immunoturbidimetry	5.23± 1.95

Table 2: Correlation of HbA1c with RBS

Method	RBS (mg%)	HbA1c (%)
Column chromatography	119.7 ±30.2	5.69 ± 1.99
Immunoturbidimetry	119.7±30.2	5.23± 1.95

Graph 1: Shows the correlation between the two methods.



The immunoturibidimetric method was faster and easier to handle so it was chosen for further study.

In the second part of the study on type II diabetic patients, microalbumin was estimated in all the diabetic and control cases in the spot urine sample. HbA1c and random blood sugar was also estimated for these subjects.

Eighty-four type II diabetes mellitus patients aged between 35-90 years were selected for this cross sectional study. The mean age of the patients were 58.64 years, out of which 56 (66.6%) were females and 28 (33.3%) males.

Table 3: Age and sex wise distribution of cases.

Age group (yrs)	Male	Female	Total
30-50	05	12	17
51-70	21	40	61
71-90	02	04	06
Total	28	56	84

Out of 84 type II diabetic patients, 60 patients had microalbuminuria, out of which 25 cases were males and 35 were females.

Table 4: Mean levels of biochemical parameters

Parameters	Controls	Cases	P value
RBS (mg%)	93.4±16.1	240.4±43.7	<0.001
HbA1c (%)	4.80±0.50	9.96±1.21	
Microalbumin (mg/dl)	8.1±3.4	108.5±36.2	

The mean levels of biochemical parameters are represented in table 4. Levels of RBS (Random blood sugar), microalbumin, and glycosylated hemoglobin were found to be higher in cases compared with controls and found to be statistically significant ($p<0.001$).

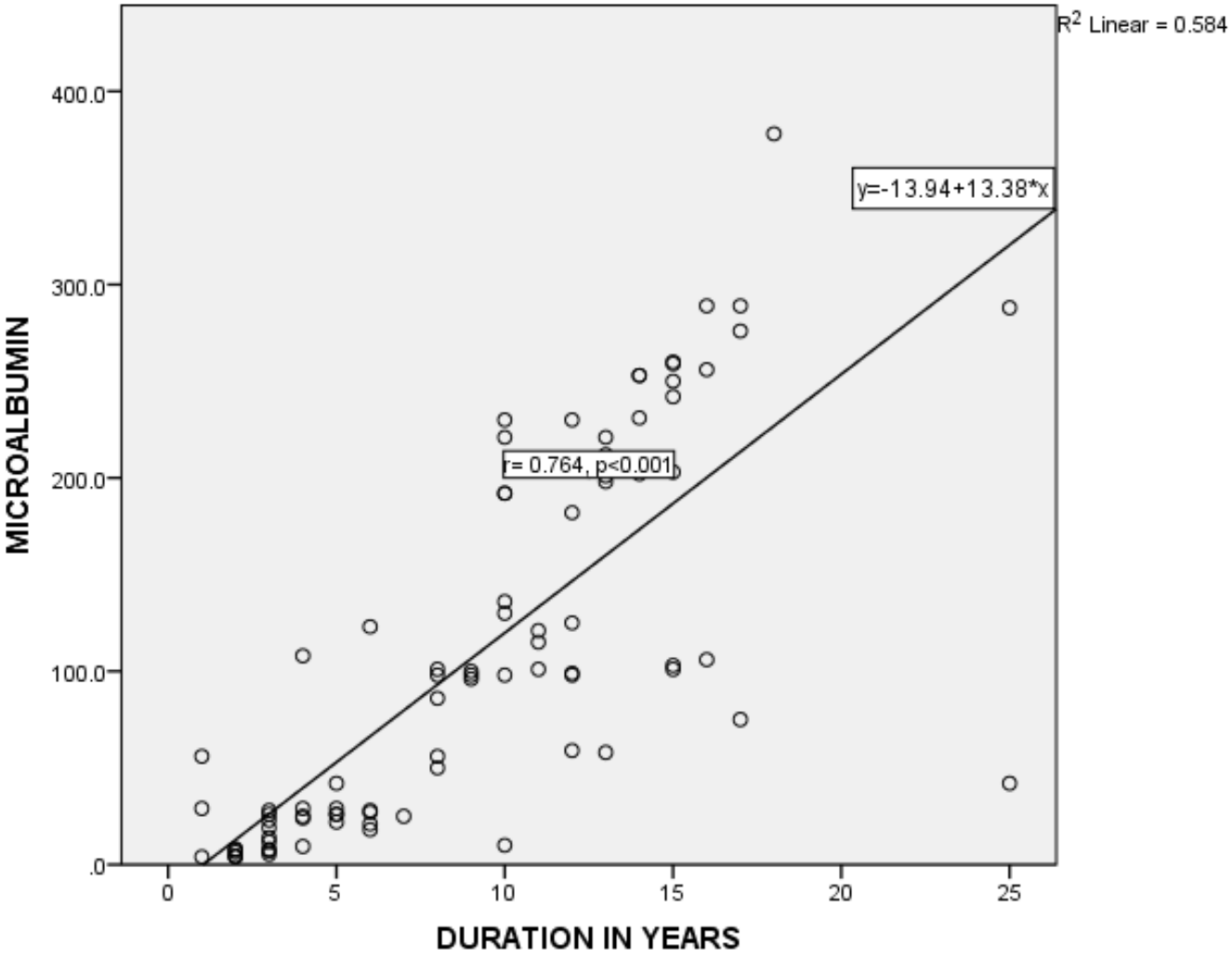
Microalbumin levels in relation to duration of type II diabetes were represented in table 5. Microalbumin levels (mg/dl) was found to be highest i.e 187.5±34.4 in diabetic subjects with duration of diabetes more than 10 years. The correlation graph of microalbumin levels to duration of diabetes is depicted in Graph 2. The correlation graphs of microalbumin levels to HbA1c in controls and cases are depicted in Graphs 3 ($r= 0.450$; $p<0.05$) and 4 ($r= 0.626$; $p<0.01$) respectively.

Forty subjects had family history of diabetes.

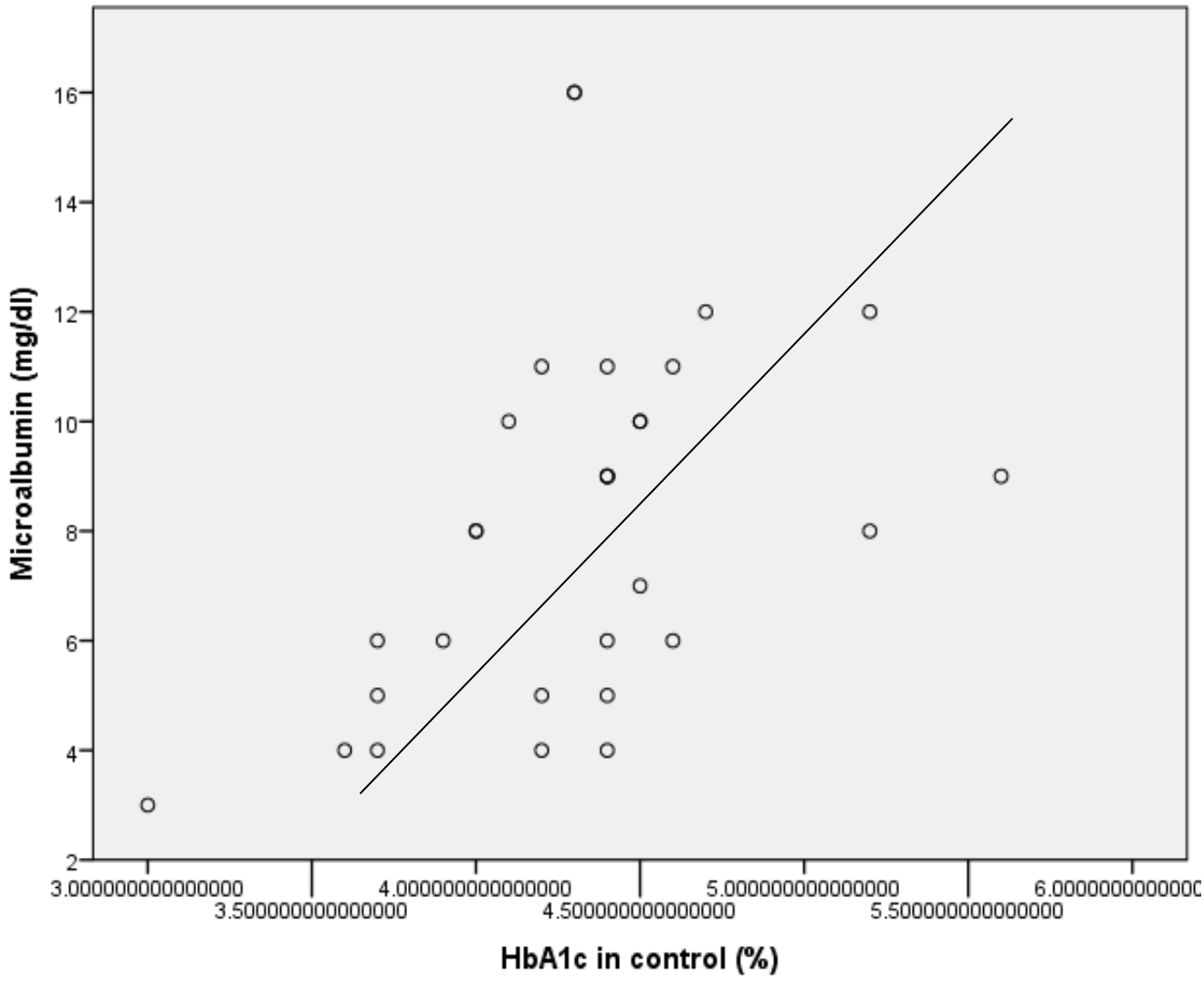
Table 5: Microalbumin and relation to duration of diabetes (in years).

Parameter duration of diabetes in years	Microalbumin(mg/dl) (Mean±SD)	P value
1-4	25.5±9.9	<0.001
5-10	82.7±16.2	
>10	187.5±34.4	

Graph 2. Correlation of microalbumin levels to duration of type 2 diabetes

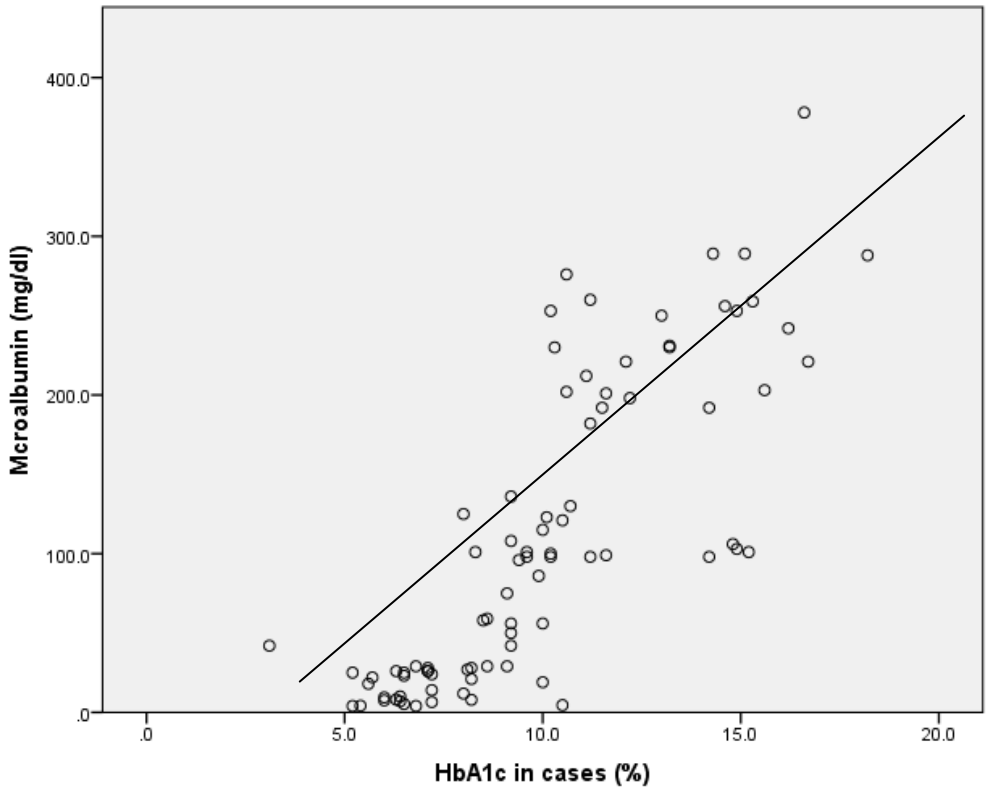


Graph 3: Correlation of microalbumin levels to HbA1c in controls



r= 0.45, p<0.05

Graph 4: Correlation of microalbumin levels to HbA1c in cases



r = 0.626, p<0.01

The result of the study shows that poor glycemic control was associated with higher proportion of microalbuminuria. Sixty of the cases i.e (71.4%) with poor glycemic control had microalbuminuria. The difference was statistically significant (P<0.012).

There was significant sex predominance, as 66.6% of females were found to have microalbuminuria compared to 33.3% of males (p=0.701).

In type II diabetes mellitus patients, microalbuminuria and glycemic control have shown a significant linear correlation with duration of diabetes (P<0.001) (Graph 2). Six of the microalbuminuric patients were smokers and, fifty four of them were hypertensive.

DISCUSSION

5.1 DISCUSSION

The prevalence of diabetes is increasing worldwide at an alarming rate (4). The patient's long-term glycemia is associated with various complications (76) and can be measured using glycated hemoglobin (77). Therefore, glycated hemoglobin measurement by laboratories should be precise and accurate. When glycated hemoglobin was measured by different methods, important differences were observed (78)

In the present study, it was found that the two methods are strongly correlated. The correlation coefficient r; was 0.905. However, immonuturibidimetric method has lower mean of HbA1c (5.23± 1.95%) compared to column chromatography (5.65±1.99%). Since immunoturbidimetric method was faster and easier to handle, it was chosen for further study.

Harris *et. al* (2003) compared four points -of -care methods with the Roche tinaqant and obtained the Pearson correlation of over 0.9 for all the four methods: DCA 2000, Nycocard, Diastat and D55. Diastat and DCA 2000 showed the best function among all the four methods. It was concluded that these two methods can be an appropriate replacement for each other, and also for the Roche method (79).

Most studies although up to date, concluded no superiority of any method (80).

Diabetes mellitus is a multifactorial metabolic disorder, related with a number of microvascular and macrovascular complications such as retinopathy, neuropathy, nephropathy, ischemic heart disease, cerebrovascular disease and peripheral vascular diseases. These complications arise as a result of the gap between the onset of the disease and clinical diagnosis of diabetes mellitus (1).

This disorder has become a major health issue in India. The burden of type II diabetes mellitus for this country (India) has been estimated to be 87 million by 2030. Type II diabetes mellitus is considered a life long disease with increasing morbidity and mortality. In addition, the disease and its complications impact a serious economic burden for the patients, their families and the society (81).

The first noticeable sign of diabetic renal disease is the presence of microalbuminuria (82), the early detection and intervention of which can delay the onset of overt nephropathy in diabetic patients (83). In order to prevent the development of microalbuminuria , blood pressure should be maintained at less than 130/80mmHg and glycemic control (HbA1c) should be maintained below 7%. HbA1c level is an important factor in the transition from normoalbuminuria to microalbuminuria and then to overt diabetic nephropathy (84).Diabetic nephropathy has been linked to elevated levels of advanced glycation end products (85).

The present study was conducted on 84 diabetic patients. Age of patients in this study ranged between 35-90 years with the mean of 58.64 years. A similar mean age was observed in several studies by Chowta NK *et. al* (2009), Kanakmani J *et. al* (2010), Maskari FA *et. al* (2008) (84,86,87).

It was found that there was a relation of glycemic control, expressed as HbA1c, microalbuminuria and duration of diabetes. Sixty out of 84 diabetic patients (71.4%) had early nephropathy. The result is found to be low compared to other studies. A study by Omar *et. al* (2015) showed that 31 out of 40 (77.5%) diabetic patients had early nephropathy (68). Other results were found to be higher than those reported by American Diabetes Association (ADA) (20-40%) (88).

In an Iranian study, it was shown that 82.7% had poor glycemic control; however, there was no correlation between HbA1c and microalbuminuria) (89). In another study by Vanelli *et. al* (2005), it was reported that higher HbA1c was associated with increasing age (90). Difference in ethnic susceptibility to nephropathy might be the likely reason for this variation.

In a study by Bruno *et. al* 2003, it was shown that 43.5% had microalbuminuria, 33.5% proteinuria, and 23% had albuminuria. Some other studies show lower levels of nephropathy (91). A study by Verma *et. al* (1994) showed prevalence of 30% nephropathy, and 50% microalbuminuria. (92).

In the present study, no statistical correlation was found between the prevalence of microalbuminuria and the age of patients which was similar to findings reported by Anwarullah *et. al* 2014 (63).

Poor glycemic control and raised blood pressure are risk factors for development of microalbuminuria. Additional risk factors include duration of diabetes and smoking (65).

Glycemic control monitoring by assessing HbA1c and urine microalbumin for nephropathy screening are two useful recommendations for the follow-up of diabetics. (65). In the present study, maximum number of patients, who had microalbuminuria, had longer duration of diabetes (10 years and above) and higher HbA1c levels (9.0-15.2%) as compared to the controls. Similar studies have been reported by other authors (93).

Micro and macrovascular complications are accelerated by hypertension (88). Microalbuminuria as suggested by Gross and coworkers (2005) tends to progress in hypertension. Serum creatinine may not be associated with microalbuminuria, however when ignored can result to irreversible renal damage. (94).

In spite of its consequences, microalbuminuria is still an unrecognized risk factor, and a large number of individuals with diabetes are not regularly screened. There are some **limitations** regarding this study.

A single morning urinary sample was estimated for microalbumin. The sample size in the two parts of the study, comparing the two methods of HbA1c estimation and correlation of HbA1c and microalbumin was small and these results cannot be applied to all individuals suffering from type II diabetes. Therefore, larger scale clinical trials to establish a correlation between HbA1c and microalbumin need to be done.

SUMMARY AND CONCLUSION

6.1 SUMMARY AND CONCLUSION

In the present study, two methods for estimating glycated hemoglobin were compared and relationship between microalbumin and glycated hemoglobin with duration of diabetes was evaluated.

- The present study was divided into two parts.
- In the first part of the study, a comparison was made between two methods available in the laboratory (immunoturbidimetric and column chromatography) for the estimation of HbA1c.
- The mean HbA1c was slightly lower for immunoturbidimetric (5.23%) method than column chromatography (5.60%) (Table 1).
- Correlation analysis between two methods was also made. The correlation coefficient was found to be 0.905 suggesting a good correlation between two methods (Graph 1).
- Correlation analysis between column chromatography and RBS and between immunoturbidimetric method and RBS was also done. The correlation coefficient was found to be 0.70 and 0.78 for the two methods suggesting a good correlation between HbA1c and RBS value for both the methods. In the second part of the study, 84 patients aged between 35 to 90 suffering from type II diabetes were selected along with 30 controls.
- HbA1c, random blood sugar and urinary microalbumin was estimated in these DM patients and in controls.
- Levels of RBS, microalbumin, HbA1c were found to be higher in diabetic cases compared with controls ($p<0.001$) (Table 4).
- Microalbumin level in relation to duration of type II diabetes was also compared. Its level was observed to be higher in diabetic subjects with duration of diabetes more than 10 years (Table 5) and there was good correlation between microalbumin and duration of diabetes ($r= 0.764$) (Graph 2).
- A correlation was also observed between microalbumin and HbA1c in controls ($r=0.405$, Graph 3) and in type II diabetic patients ($r=0.626$, Graph 4).

- Microalbumin was found to be higher in (71.4%) of cases with poor glycemic control compared to (28.5%) of cases with good glycemic control.

Being a developing country, there is a great need that microalbumin and HbA1c testing should be done in both, newly diagnosed as well as already diagnosed type II diabetic patients as early markers of nephropathy. This is because the prevalence of microalbuminuria in diabetic patients is found to be high. Therefore, patients and physicians should give very high priority to improving glycemic control sufficiently to maintain glycated hemoglobin values below 7%. If this can be achieved, the number of patients in whom microalbuminuria develops would decline substantially, and in turn, lower the number of patients who develop end-stage renal disease.

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PATIENT'S PROFORMA

NAME			**MRD NO.**		
			GENDER		
SMOKING (YES/NO)		**NO OF CIGARETTES SMOKED IN DAY**		**NO OF TIMES SMOKED IN WEEK**	
FAMILY HISTORY			**DM/HT**		
DIABETES (YES/NO)			**DURATION OF DIABETES**		
TREATMENT OF DIABETES	**INSULIN**				
	ORAL				
	AYURVEDIC				
	DIET				
	HOMEOPATHY				
HYPERTENSION (YES/NO)			**DURATION OF HYPERTENSION**		
DRUG NAME			**DURATION**		
ANY OTHER ABNORMALITIES					

REPORT

TEST	RESULT	UNIT
RBS		60-140mg/dl
Micro albumin		<300mg/dl
Glycated HB		<7%