Association Between Urinary IgG and Relative Risk for Factors Affecting Proteinuria in Type 2 Diabetic Patients

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Abstract Abnormal glomerular permeability is the primary step towards the glomerulosclerosis. The progression rate of glomerulosclerosis is proportionate to abundance and severity of lesions created at incipient stage, which is reflected as proteinuria even though eGFR remains in the normal range. Therefore, there is a current need to find out the association between relative risks for the factors leading to proteinuria. The relations could be more informative, if it is with respect to the macromolecules like “IgG” excretion in urine. Type 2 diabetic patients were selected for this study with eGFR > 75 ml/min/1.73 m² and grouped into four quartiles based on UIgGCR. The markers of key factors affecting progression of proteinuria were estimated through biochemical tests. The impact of these markers on proteinuria was accessed by applying multinomial logistic regression. The adjusted odds ratio for the UGAGCR was 1.186 (95 % CI: 1.061–1.327) $P < 0.003$ in highest quartiles of UIgGCR, followed by odds ratio for markers of collagen catabolism 1.051 (95 % CI: 1.025–1.079) $P < 0.001$, and USACR 1.044 (95 % CI: 1.013–1.077) $P < 0.006$ respectively. The marker of glycation, i.e., glycated hemoglobin showed the highest odds ratio 5.449 (95 % CI: 1.132–26.236) $P < 0.035$. In addition, odds for the systolic blood pressure was observed 1.387 (95 % CI: 1.124–1.712) $P < 0.002$. The higher odds inform and could help to discriminate the diabetic patients

with fast progressive diabetic nephropathy. The study describes critical relationship between the urinary excretion of IgG and factors leading to proteinuria in type 2 diabetic patients.

Keywords Diabetic nephropathy · Urinary glycosaminoglycans · Urinary sialic acid · Proteinuria · Glomerular basement membrane

Abbreviations

UGAGCR Urinary glycosaminoglycan creatinine ratio
UHPCR Urinary hydroxi-proline creatinine ratio
USACR Urinary sialic acid creatinine ratio
UIgGCR Urinary IgG creatinine ratio

Introduction

Type 2 diabetes is rapidly increasing with change in lifestyle in developing countries, recently normoalbuminuric patients have shown 9.47 % prevalence of end stage diabetic nephropathy [1], which is more prevalent in urban than in rural area [2]. The major threats for development of diabetic nephropathy is uncontrolled blood sugar which directly and indirectly leads to metabolic disturbances [3, 4] in addition, the Steno hypothesis suggested that the loss of anionic charge from basement membrane leads to diabetic microangiopathy [5]. Charge, size, and shape of molecules are three hinderic properties which restore them into plasma from urine; plasma albumin frequently appears in urine with the subtle change of these factors. In addition these factors are frequently influenced by presence of other secondary complications. Natural glycation is an essential process which maintains the functional and structural

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integrity of several biomolecules on Glomerular basement membrane (GBM). GBM bears a layer of glycosaminoglycans (GAGs) which provides the first barrier for the plasma filtration process, which is suggested to be lost in incipient diabetic nephropathy [6, 7]. Moreover, Sialic acid moiety caps are suggested to provide negative charge to the cell surface. Which are potentially able to alter the functionality of membrane receptor and glycoprotein conformations. The free fraction of sialic acid which increases in response of increased stress is filtered through GBM and not reabsorbed by tubular epithelial cells therefore serves as a marker for loss of charge selectivity [8]. Of note, studies have shown that the extensive glycation and desialation process, of GBM proteins at high blood pressure leads to dismantle of GAGs layer which are excreted into the urine. The bared GBM has been suggested for the increased susceptibility for renal fibrosis as it promotes invasion of numerous plasma molecules into GBM, which further promotes invasion of cells [9, 10]. These pathways create kidney lesions without microalbuminuria, leads to increase collagen catabolism and excrete hydroxyproline peptides in urine, thus it reflects deterioration of renal function [11, 12]. In addition, deteriorated glomerular endothelial surface layer precisely Podocytes cells detachment leads to increase urinary excretion of macromolecules [13, 14], which is further facilitated by nonenzymatic glycation of proteins [15]. Therefore urinary excretions of macromolecules are supposed to relate with deterioration of glomerular endothelial surface layer and its abnormal permeability [16, 17]. And there is a hope for urinary IgG as better marker to severe lesion at early stages of diabetic nephropathy. The present study aimed to reveal impact of factors affecting renal deterioration among the patients in increasing order of loss of size selectivity using urinary IgG as biomarker, and to find better alternative for the recently raised doubt on urinary albumin as risk predictor of nephropathy in type 2 diabetic patients [18, 19].

Materials and Methods

Study Design and Participants

The cross section study included type 2 diabetic patients from the western region of India. Patients were instructed to attend the OPD at morning session after overnight fasting. Blood and urine samples were collected with written and informed consent as per the approved protocol of an ethical committee of S. K. Hospital and P. S. Medical Collage, Karamsad, Gujarat, India. Most of the patients were from semi urban area. None of the patients was immunologically compromised regarding infection, lesions like diabetic foot and chronic illness or overt proteinuria.

Patients were taking single dose of hypoglycemic agents and they were found of discontinuation of medication. Only those type 2 diabetic patients were selected who were having renal functional eGFR > 75 ml/min/1.73 m² calculated by MDRD equation.

Sources of Materials

Consumable item like blood and urine collection vials were purchased from BD Vacutainer® and Tarsons Products Pvt. Ltd. respectively, Amicon ultra filtration concentrating units were purchased from Millipore. All chemicals used in current study were of analytical grade and purchased from Sigma or Merck Chemicals Ltd. Kits for biochemical analysis were purchased from eve's diagnostic Baroda, India. Folin reagent was purchased from Merck chemicals and standard total protein from Transasia Bio-Medical Ltd. Daman, India. Anti Human IgG, HRP conjugated antibodies and TMB for ELISA were purchased from Bangalore Genei Pvt. Ltd. India.

Laboratory Measurements

Plasma parameters were analyzed by laboratory protocols. Plasma levels of blood sugar, triacylglycerol, cholesterol and HDL-cholesterol were assayed by commercially available kits. Urinary albumin was measured by IMMULITE auto analyzer. The modified Jaffe's method was used to measure plasma and urine creatinine concentrations [20], Glycated hemoglobin [21], Protein estimation was done by Lowry et al. [22] method. The urinary protein/peptide bound hydroxi-proline content was estimated by previously published method [23]. In brief urinary proteins/peptides were isolated from urine by filtering 0.22 μ m filter to remove debris followed by Amicon ultra filtration concentrating unit's cut-off value 2.0 kd to remove salts and washed with deionized distilled water. Plasma and urinary IgG were measured by previously established protocol in our lab published elsewhere [15], Plasma and urinary sialic acid content was estimated [24], Urinary glycosaminoglycans were determined and bovine kidney heparan sulfate was used as standard [25], Urinary biomarkers were expressed in terms of the creatinine ratio. The estimated GFR was calculated from MDRD equation [26].

Statistical Procedure

Statistical evaluation was performed using the independent sample t test. The demographic and clinical data were expressed as mean $\pm$ SD or median (range). In addition, all the patients were subjected to segregation, into four quartiles on the basis of their urinary IgG creatinine ratio (UIgGCR). The first quartile was set as reference for the

analysis of data applying multinomial logistic regression. The odds ratio was calculated for the variance in rest of the three quartiles and further adjusted for traditional co-founders age, gender and smoking habits. All statistical analysis was done using SPSS 17 software.

Results

Clinical characteristics of the type 2 diabetic patients listed in Table 1, as compared with controls, type 2 diabetic patients were characterized by increased glycemic indices, increased levels of plasma cholesterol, triglycerides and HDL. A significant change in BMI, diastolic blood pressure was also observed when compared with controls.

In all four quartiles, biomarkers related to renal function have shown clear discrimination from control subjects, more specifically urinary sialic acid creatinine ratio (USACR), urinary glycosaminoglycan creatinine ratio (UGAGCR), and urinary hydroxi-proline creatinine ratio (UHPGR) seem to be independent predictors of progressive diabetic nephropathy which possible leads to increase number of shunt like pores; the values are shown in Table 2. Multinomial logistic regression analysis for the factors affecting proteinuria, has shown in Table 3. The USACR has shown a subtle change in adjusted odds ratio from 1.025 (95 % CI: 1.000–1.050) $P < 0.049$ in second quartile, to fourth quartile of UIgGCR, 1.044 (95 % CI: 1.013–1.077) $P < 0.006$ seems to be key impact on UIgGCR. Moreover, we also analyse the effect of UGAGCR to assort the IgG in urine, based on different quartiles of UIgGCR. The adjusted odds ratio for UGAGCR

Table 1 Clinical characteristics of type 2 diabetic patients

	Control	Diabetic patients
<i>n</i>	50	352
Age (years)	47 ± 7	51 ± 10*
Men %	56	54
Duration (years)	–	12.42 ± 5.82
BMI (kg/m ²)	23.48 ± 2.14	26.67 ± 3.92*
Smoking (%)	16	13
Fasting (mg/dl)	84.30 ± 5.24	148.32 ± 34.75*
PP2BS (mg/dl)	117.46 ± 6.64	229.49 ± 41.43*
Glycated Hb %	5.31 ± 0.45	7.9 ± 1.0*
SBP (mmHg)	114 ± 10	125 ± 11*
DBP (mmHg)	74 ± 7	78 ± 7*
Cholesterol (mg/dl)	141.89 ± 40.36	196.82 ± 49.38*
Triglycerides (mg/dl)	122.09 ± 41.27	160.91 ± 60.08*
HDL (mg/dl)	47.54 ± 12.81	48.88 ± 13.61*
LDL (mg/dl)	109.68 ± 30.99	147.53 ± 45.77*

Variables are presented as either mean ± standard deviation

* P values < 0.05 denote statistical significance

Table 2 Biomarker related to renal function

Clinical parameter	Control	First quartile	Second quartile	Third quartile	Fourth quartile
Plasma protein (g/dl)	6.87 ± 0.52	6.62 ± 0.79	6.53 ± 0.80	6.33 ± 0.84	5.92 ± 0.79
Plasma IgG (mg/dl)	844.85 ± 30.00	1,138.6 ± 78.10	1,077.8 ± 67.36	1,063.2 ± 70.96	996.76 ± 74.71
Urinary IgG (mg/g creatinine)	1.8 (0.16–3.33)	9.81 (1.18–14.93)	20.10 (14.99–25.09)	33.27 (25.11–45.57)	69.45 (45.82–98.64)
Plasma albumin (g/dl)	4.12 ± 0.32	3.95 ± 0.79	3.76 ± 0.97	3.84 ± 0.97	3.51 ± 0.10
Urinary albumin (mg/g creatinine)	2.89 ± 1.49	11.49 ± 11.80	27.94 ± 16.32	90.89 ± 88.68	122.46 ± 81.98
Plasma sialic acid (mg/dl)	52.81 ± 6.87	63.41 ± 7.23	65.05 ± 8.38	69.29 ± 8.90	72.05 ± 11.65
USACR (μg/g creatinine)	35.45 ± 19.90	79.94 ± 33.09	115.62 ± 43.92	132.32 ± 35.07	153.10 ± 44.86
UGAGCR (μg/g creatinine)	47.91 ± 11.48	50.10 ± 10.53	50.11 ± 10.54	64.51 ± 18.58	81.02 ± 26.62
UHPGR (ng/g creatinine)	197.97 ± 105.23	399.51 ± 97.67	913.49 ± 232.99	1,349.71 ± 517.75	5,019.19 ± 2,469.89

Variables are presented as either mean ± standard deviation. Urinary IgG was presented as median (range)

Table 3 Multinomial logistic regression analysis for the factors affecting proteinuria, adjusted with gender, age and smoking

	First quartile Reference	Second quartile OR (95 % CI)	Third quartile OR (95 % CI)	Fourth quartile OR (95 % CI)
USACR	1	1.025 (1.000–1.050)	1.033 (1.007–1.060)	1.044 (1.013–1.077)
<i>P</i> value		0.049	0.013	0.006
UGAGCR	1	1.077 (0.969–1.196)	1.138 (1.021–1.268)	1.186 (1.061–1.327)
<i>P</i> value		0.169	0.019	0.003
UHPGR	1	1.046 (1.019–1.073)	1.049 (1.022–1.076)	1.051 (1.025–1.079)
<i>P</i> value		0.001	0.001	0.001
Glycated hemoglobin	1	3.453 (0.865–13.782)	3.985 (0.952–16.674)	5.449 (1.132–26.236)
<i>P</i> value		0.079	0.058	0.035
Systolic blood pressure	1	1.139 (0.940–1.380)	1.249 (1.025–1.523)	1.387 (1.124–1.712)
<i>P</i> value		0.184	0.028	0.002
Diastolic blood pressure	1	1.023 (0.791–1.325)	1.047 (0.803–1.365)	1.042 (0.788–1.378)
<i>P</i> value		0.861	0.734	0.772
Duration of diabetes	1	1.034 (0.877–1.218)	1.000 (0.838–1.194)	1.075 (0.860–1.344)
<i>P</i> value		0.691	0.997	0.523

OR odds ratio, CI confident interval

Quartiles were classified on the basis of UIgGCR of type 2 diabetic patients

increases from 1.077 (95 % CI: 0.969–1.196) to 1.186 (95 % CI: 1.061–1.327) $P < 0.003$ from second to fourth quartile respectively. From these results it seems that the increased in odds for UGAGCR is the pivotal step in glomerulosclerosis and creation of shunt like pores. To determine the speculated contribution of renal matrix in occurrence of high UIgGCR; we analysed marker of collagen catabolism urinary peptide bound hydroxi-proline UHPGR in addition to marker of glycation, hypertension and duration of diabetes to a multivariable logistic regression adjusted for age, gender and smoking habits of patients. In this analysis the first quartile was set as reference and rest three quartile were compared. As the adjusted odds ratio for UHPGR increases from 1.046 (95 % CI: 1.019–1.073) to 1.051 (95 % CI: 1.025–1.079) $P < 0.001$ from second to fourth quartile of UIgGCR as shown in Table 3, this 11 % change in odds ratio for UHPGR suggested to increase porosity of glomerular membrane significantly. We observed a 2–18.6 % change in log odds ratio when unadjusted through second to fourth quartile these results deduced that age, gender and smoking have great influence on odds for UHPGR throughout the quartiles in increasing order.

The adjusted odds ratio for glycated hemoglobin increased from 3.453 (95 % CI: 0.865–13.782) to 5.449 (95 % CI: 1.132–26.236) $P < 0.035$ as shown in Table 3, which clearly indicates that the appearance of trace amount of macromolecules in urine is strongly facilitated by increased glycation. We further observed that the odds for glycation is frequently increased upon adjustment, which shows that glycation control is affected by age, gender and

smoking. Systolic blood pressure has shown the higher significant impact on UIgGCR where as diastolic blood pressure remains statistically insignificant. The odds ratio for the duration of diabetes in all quartiles was insignificant which indicates that patients from first through second and third up to fourth quartiles were indistinguished by duration, therefore observed odds were statistically insignificant.

Discussion

Kidney is composed of four types of cells: the parietal epithelial cells builds Bowman's capsule, the outermost layer of the glomerular filtration barrier is wrapped by podocytes, the fenestrated endothelial cells coated with glycocalyx which faces towards the blood flow and the last type i.e., mesangial cells which connect the capillary loops. The filtration process is accomplished with the equal contribution of these cells dedicated for their own assignments [27, 28]. Kidney potentially separates plasma constituents into water, solute, cells and filtrate known as primary urine, which is further reabsorbed selectively. The GBM restricts the passage, on the basis of molecular weight, charge, and shape of the macromolecule, for this purpose strong coordination, movement and cross-talk between these four constituent cells are necessary [27]. Interruption of these processes has been suggested for the development of diabetic nephropathy. Our finding of adjusted odds ratio for USACR increases in chronological order among UIgGCR quartiles, might related to the process of interruption in

communication as numerous key proteins on GBM are heavily sialated. Recently glomerular endothelial cells have been reported to modulate the podocyte function and interrupted cross-talk between the cellular compartments of the glomerular capillary walls and altered barrier of plasma protein filtering units [29]. This ultimately leads to macromolecules in the urine [16] followed by intact podocyte loss in type 2 diabetic patients [30]. Moreover, sialic acid is also present on podocytes and it is also an integral part on the podocalyxin, at glomerular basement membrane [16]. In our previously published work, we reported that the sialic acid in diabetic patients is also a good diagnostic marker for early diabetic nephropathy. The release of sialic acid in urine represents bared structural moieties on GBM; this further promotes the release of GAGs into urine potentially leading to peel of GBM. Endothelial glycocalyx damage is further affected by hyperglycemia and microalbuminuria [31]. In addition to this, podocytes have been reported for least regeneration, therefore those diabetic patients who frequently suffer glycemic variations are supposed to susceptible for shunt like pores creation by podocyte foot effacement. Moreover, loss of podocyte has been reported in type 2 diabetics [30] irrespective of microalbuminuria, this could be why we observed higher odds ratio 1.186 (95 % CI: 1.061–1.327) $P < 0.003$ for UGAGCR in fourth quartile of UIgGCR as shown in Table 3. It is important to note that the increased UIgGCR reflects GBM porosity and pathology. We did not find any significant correlation between decreased eGFR and urinary sialic acid concentration. Urinary sialic acid and urinary GAGs are suggested to play an important role in progression of adherence surface leading to GBM deterioration and additional porosity. Furthermore it was observed that higher quartiles of UIgGCR shown highest odds ratio 1.186 (95 % CI: 1.061–1.327) for UGAGCR, which was lower than 1.044 (95 % CI: 1.013–1.077) USACR. Which indicates that UIgGCR is highly influenced by GBM GAGs content and the similar results have been observed in type 1 diabetic patients [32], which suggests few common pathways exist in progression of diabetic nephropathy in type 1 as well as in type 2 diabetic patients.

Long term hyperglycemia as well as frequently occurring short term glycemic fluctuations activates multiple pathways [33]. Thus renal remodulation and collagen turnover takes place at incipient stages of type 2 diabetic nephropathy [34, 35]. This leads to deprivation of integrity leading to increase mesangial expansion followed by nodular sclerosis (Kimmelstiel–Wilson lesions) [36]. It was observed that in diabetic nephropathy, glomerular injury is leading factor and nephropathy starts from ultrafiltrate of a variety of cytokines, chemokines and growth factors [37, 38]. Afterwards, these factors potentially mediate inflammatory, fibrogenic reactions and the synthesis of

extracellular collagenous matrix [38, 39], the loss of immature or semi-digested collagen peptide in urine leads to severe scar [40]. The low odds ratio with high significance can be explained as, first; the accumulation of collagen in advanced nephropathy is characterized by increased synthesis and decreased proteolysis. Secondly; high glucose has been reported for decreased activity of proteolytic enzymes. In addition, renal fibrosis has been seen in patients with serum creatinine >2.5 mg/dl. It has been seen that glomeruli with epithelial injury and focal capsular adhesions are prone to detach from their tubules resulting into atubular glomeruli [41], leading to frequent loss of macromolecules in urine as they are not reabsorbed by tubular epithelia. This is why we observed a little change in odds ratio for UHPCR at higher statistical significant level: 1.046 (95 % CI: 1.019–1.073) to 1.051 (95 % CI: 1.025–1.079) $P < 0.001$ from second to fourth quartile of UIgGCR shown in Table 3.

It has also been observed that glycation of protein increases its acidic nature [42]. At acidic pH, binding of protein to FcRn receptors is higher when compared to physiological pH [43]. The interactions between GAGs and proteins are highly dependent on the conditions of their local micro-environment, such as cation content, their concentration and pH [7]. This is why high glucose has been suggested to increase tubular apoptosis in diabetes, independent of systemic hypertension [44]. Moreover excessive receptor-mediated internalization of proteins at the tubular region generates excessive H_2O_2 and ammonia [45]. We have previously reported that the prolonged hyperglycemia leads to nonenzymatic glycation of proteins, which ultimately loses their negative charge leading to increase urinary excretion of macromolecules like IgG [15, 46]. Size and shape of the macromolecule are next hindrance for its appearance in urine, hyperfiltration was shown to be an independent predictor of diabetic nephropathy [47], and increase in renal blood flow and glomerular filtration rate, which is seen in typically at the onset of diabetes mellitus may be suggested to initiate diabetic nephropathy [48]. Numerous researchers had suggested an association of hypertension and increased amount of microalbumin in urine, at the time of onset of diabetes the excretion is less favored for IgG like macromolecules. Moreover, glycation of small molecules might result into rapid loss of surface charge where as glycation of macromolecules might lose the charge but increase steric hindrance of size. Therefore, we suggest urinary excretion of IgG predicts a serious lesions and lesser extent of bias influence. We observed adjusted odds ratio 1.387 (95 % CI: 1.124–1.712) $P < 0.002$ for systolic blood pressure, it is concluded that hypertension potentially imparts in macroproteinuria.

Numerous studies suggest administration of sulodexide (a purified GAG) reduces microalbuminuria in type 2

diabetic patients [49–51] but attention has not been paid to the urinary excretion of macromolecules. Reduction in microalbuminuria may be a resultant of restoration of charge selectivity. Whereas restoration of macromolecules in urine reflects depletion of shunt like pores or widened scars. The present study includes numerous limitations and strengths. The first limitation of this study is the single-point measurement of these biomarkers. Secondly this study lacks of biopsy proven data. Furthermore, because the study was an observational analysis, causal links and relations/associations are quite possible. However adequate adjustments were used to account for overt bias. We were careful to interpret and explain our results in terms of associations and not causal effects due to the potential for residual confounding. The medical records of patients were screened thoroughly; we believe that these data are representative of the patient's long-term values. On other hand our study has several important strengths. The combined population at rural and urban area allowed us to identify an adequate number of representative patients with diabetes and hypertension to fully explore progression through increasing stages of nephropathy in a real-world population. Another strength of this study is that, we do not dichotomize continuous variables data that gives an additional impact on accuracy. In addition, our study might help in automation of these biomarkers with accurate relation to the mechanism of injury.

Conclusion and Future Directions

Presently no of the biomarker is available which perfectly diagnose progressive incipient diabetic nephropathy. On an average 7 % of diabetic patient's already suffer from diabetic nephropathy when they diagnosed first [52]. Some of the diabetic patients develop diabetic nephropathy in short duration, due to variation in response to drugs, variation in life style and environmental factors ect. Because of diabetic nephropathy originates heterogeneously therefore urinary albumin is doubtful biomarker where as an increment in UI-gGCR index is not virtual and strictly associated with numerous factors, which maintain physiochemical hemostasis. Therefore a level of urinary IgG represents the extent of serious lesions in kidney. We suggest UIgGCR might be helpful for selecting patients on higher risk of diabetic nephropathy. Further investigations are needed to answer whether the treatments of type 2 diabetes with administration of ACE inhibitors/ARB, sialic acid and sulodexide ect. reduces only microalbuminuria or helpful in healing of shunt like pore.

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