Prediction of Renal Injury Risk by Expressions of KIM-1 and NGAL in Type 2 Diabetic Nephropathy

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Authors' contributions

This work was carried out in collaboration between both authors. Author VVK designed the study, wrote the protocol, experimental process and wrote the first draft of the manuscript. Author KSY managed the literature searches, analyses of the study performed. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Background: About 10 to 40% Type 2 diabetes (T2DM) and 30% Type 1 diabetes (T1DM) suffer from kidney failure increases huge financial burden for care for patients [1]. Patients with uncontrolled diabetes prone to end-stage renal disease (ESRD) which required kidney transplantation, haemodialysis or peritoneal dialysis which adds psychological & financial burden. Early kidney injury can be prevented by evaluating gene expression (KIM1, NGAL) in T2DM with microalbuminuria.
Methodology: This study includes 241 subjects (118 male, 123 women, and age ranges 30-70 years, distributed in two groups; 30-45 years and 45-70 years) were included after screening for T2DM by measurement of blood glucose in fasting, post-prandial, glycosylated haemoglobin and micro albumin in urine. Subjects were randomised after written consent; subject examined by physician and enrolled as per inclusion/exclusion criteria. Categorization of subjects in three study groups were done on the basis of T2DM duration 3-5 years, Glycosylated haemoglobin level (HbA1c) ≥ 7.0% with fasting blood glucose ≥126 mg/dl) and micro albuminuria in study group.

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Equal numbers of age and sex matched healthy volunteers enrolled in control group. Blood samples were processed for other renal parameters &rt-PCR to check expressions of KIM1 and NGAL.

Results: In study groups all renal parameters are within normal range except albumin creatinine ratio (p<0.012) & e-GFR (p<0.000). Other parameters showed marginal significance within and between the groups. KIM-1 and NGAL showed high degree of significance (p<0.000).

Conclusion: Biochemical renal parameters are not enough to identify risk of DN even in microalbuminuria. Early detection of gene expressions of KIM1 and NGAL may help to evaluate the status of kidney functions which help to prevent morbidity & mortality of kidney due to diabetic nephropathy. Extensive study in large population size was recommended.

1. INTRODUCTION

Recent estimates by the National Institutes of Health indicate that diabetes represents the single largest cause of ESRD [1]. Today it has become the single most common cause of (ESRD) in the entire world. T2DM subject undergoing dialysis consumes significantly more financial resources than those with non-diabetic ESRD. In addition, T2DM patients do poorly on dialysis and have an excess mortality. Clinical management and therapeutic intervention in stage of DN has major role in prevention and progression of DN to ESRD. DN is typically defined by microalbuminuria, i.e., renal albumin excretion more than 300 mg in 24 hrs collection (urine dipstick positive) and abnormal renal function (increase serum creatinine or GFR). Clinically nephropathy with T2DM was characterized by progressive increase in proteinuria and decline in GFR. There are several biomarkers in use to detect kidney damage. Conventional biomarkers for kidney damage include glomerular filtration rate (GFR), plasma creatinine, blood urea nitrogen (BUN), urinary micro-albumin excretion rate and several urine qualities such as proteinuria and hematuria as well as liver function test, glycated hemoglobin, RBC indices and urine routine. However these routinely used parameters to evaluate kidney function are nonspecific and insensitive, hence new specific markers are being developed [2].

Poorly sensitive methods are failed to recognized early detection of DN, these patients has greater chances of ESRD. Use of novel biomarkers has potential to detect early DN progression. Several novel biomarkers of kidney injury have been shown to increase in the urine & plasma of individuals with diabetes at early stage [3].

DN develops in, at the most, 40% of diabetic patients, even in controlled blood glucose concentration for long period. This observation raised the concept that subsets of patients have an increased susceptibility to DN. Furthermore, epidemiological and familial studies have demonstrated that genetic susceptibility contributes to the development of DN in patients with T2DM [4]. The main potentially modifiable DN initiation and progression factors in susceptible individuals are sustained hyperglycaemia and hypertension [5]. Other putative risk factors are GFR, smoking, dyslipidemia, proteinuria levels, and dietary factors, the main potentially modifiable DN initiation [6,7]. A higher proportion of individuals with T2DM are found to have microalbuminuria and overt nephropathy shortly after the diagnosis of their diabetes, since diabetes is actually present for many years before the diagnosis was made and also due to the fact that presence of albuminuria may be less specific for the presence of DN [8]. Without specific interventions, 20-40% of T2DM patients with microalbuminuria progress to overt nephropathy, but after 20 years onset of overt nephropathy, only 20% will have progressed to ESRD. Once the GFR begins to fall, it differs from one individual to another, but overall, they may not be substantially different between T1DM patients and T2DM Patients [9]. The prediction of microalbuminuria is an indication for screening for possible vascular disease and aggressive intervention to reduce all cardiovascular risk factors (e.g., reducing LDL cholesterol, antihypertensive therapy, cessation of smoking, institution of exercise, etc.). Additionally, there is some preliminary indication that suggests lowering of cholesterol also lowers the level of proteinuria [10].

Routinely used investigations for kidney injury are serum creatinine & blood urea. These are conventional biomarkers have several disadvantages. Serum levels of creatinine may only change after about 50-60% of the kidney function has been lost. Altered levels of serum creatinine take time to establish & make it impossible to detect acute kidney injury early.

There is utmost need to know highly sensitive biomarkers for the early detection of. Inadequate tools are failed to recognize early detection of DN at an early stage. So it was postulated that KIM1 and NGAL may help to identify the risk of DN.

Kidney Injury Molecule (KIM1) is a type-1 trans-membrane glycoprotein not detected in normal kidney, tissue or urine but it appears at high levels after ischemic or toxic kidney injury [11]. (KIM-1) is a promising biomarker for kidney damage. It is structurally related to the immunoglobulin gene family [12].

There are many reasons to consider that KIM-1 may be released into the circulation after kidney proximal tubule injury. In injury, tubular cell polarity is lost, such that KIM-1 may be released directly into the interstitium. Further, increased trans-epithelial permeability after tubular injury leads to leak of tubular contents into the circulation [13]. Also, altered micro vascular permeability is an important contributor to the pathophysiology of kidney injury [14].

NGAL is a member of the lipocalin family of proteins and is expressed only at very low levels in several human tissues, including kidney [15]. Therefore NGAL protein has been extensively studied in different models of acute kidney injury (AKI). In numerous studies, the increase of NGAL in urine and serum has been a good predictor of a onset of AKI, especially anticipating the increase in serum creatinine [16-20]. Literature survey revealed that NGAL could be a potential marker of CKD [21,22].

Human NGAL was originally identified as a novel protein isolated from secondary granules of human neutrophils [23,24]. NGAL mRNA is normally expressed in a variety of adult human tissues, including bone marrow, uterus, prostate, salivary gland, stomach, colon, trachea, lung, liver and kidney [25].

2. MATERIALS AND METHODS

Present research conducted at Department of Biochemistry, Dr D. Y. Patil University, Navi Mumbai. Patients referred to Diabetic clinic OPD were recruited in this study. The enrolled patients were randomly selected & distributed into three different groups; subjects of T2DM between ages 30-45 years; subjects of T2DM between 45-70 years and healthy volunteers (Non-diabetic) between 30-70 years.T2DM of diabetes duration between 3-5 years, HbA1c ≥ 7.0%, pre-prandial blood glucose (FBS-126 mg/dl), post-prandial glucose (PPBS-200 mg/dl) and microalbuminuria (30-300 mg/dl) were included in this study. Subjects satisfying above criteria but suffering with chronic conditions were excluded from the study. Other renal parameters (blood urea, serum creatinine, urine creatinine calcium and uric acid was measured and e-GFR, albumin-creatinine ratio were calculated from previously collected serum & urine samples. 3 ml whole collected for gene expression separately. All biochemical renal parameters were measured by Dade Dimension dry chemistry auto-analyser (Roche Diagnostics), isolation and amplification of m-RNA was performed by "One Step Prime Script RT-PCR (Perfect Real Time)" designed by using Taq Man® probe. KIM-1 and NGAL expressions were measured from buffy coat, a concentrated leukocyte suspension of whole blood. Procedure for preparing buffy coat from whole blood; whole blood was collected in a blood collection tube containing anticoagulant EDTA with aseptic precautions. Added 1 part phosphate buffered saline (PBS) + 2% FBS (Fetal bovine serum) to 1 part fresh whole blood. Reaction mixture centrifuged at room temperature and 200 x g for 10 minutes. Concentrated leukocyte band called buffy coat was removed carefully. Contamination in the buffy coat can be minimized by avoiding RBC lees band with minimum plasma.

3. RESULT

Screening parameters used for diagnosis of microalbuminuria & non- microalbuminuria showed significant P value. Results are expressed as means ± SD and standard error. Statistical calculations were performed using the R software package. Means in control and type 2 diabetic groups were compared.

Statistical analysis (descriptive and post hoc) of Tables 1 & 2 showed significant difference in P value of HbA1c and urine creatinine. Though GFR is considered as gold standard parameter for renal assessment but in early stage of DN it has less clinical usefulness. Observation of e-GFR in this study was supported by Nielsen SE, et al. study [26].

In this study it was found significant P-value (p <0.000) in all study groups. High degree of significance was found in both KIM-1 and NGAL (Table 3) despite all routine renal parameters reported within reference interval. KIM1 and NGAL m-RNA expressions were measured from a plot (Figs. 1 & 2) which is mentioned in Table 3.

4. DISCUSSION

KIM1 is a type 1 trans-membrane protein which is exclusively and abundantly expressed in damaged kidney cells. The ectodomain of KIM-1 is shed into urine and easily detectable. According to Waanders F, et al. [27] KIM-1 expressions are measurable within a day after the onset of kidney damage. It is a marker for acute and chronic kidney disease. It was observed that blood KIM1 expressions were unregulated in this study. Similar results were demonstrated by Markus Alter, et al. [28] in experimental diabetic nephropathy study, which shows proximal tubular injury in rodent. In contrast, blood KIM-1 was reported raised shortly after proximal tubular injury [29]. Another study

Table 1. Descriptive analysis of renal parameters, Microalbumin (MALB), Glycosylated hemoglobin (HbA$_1$C) and Albumin-creatinine ratio (ACR) within groups

Groups / Parameters	Control Mean	SE	45 years and less Mean	SE	More than 45 years Mean	SE
Glycosylated haemoglobin	5.6	0.052	8.0	0.157	8.0	0.129
Blood glucose (F)	96	0.806	147	4.638	156	5.941
Blood glucose (PP)	108	0.921	175	4.242	197	7.696
Micro-albumin	14.13	0.401	235.28	5.970	263.37	9.462
Urine creatinine	60.99	4.335	121.06	9.231	134..65	13.960
Albumin/Creatinine ratio	0.44	2.113	3.35	3.556	3.35	2.623
Calcium	9.4	0.064	9.6	0.069	9.6	0.057
Blood urea nitrogen	10	0.284	10	0.237	11	0.335
Uric Acid	4.8	0.112	5.0	0.203	5.3	0.139
Serum creatinine	0.79	0.02	0.716	0.019	0.854.	0.023
e-GFR	100	2.46	94	114	90	2.077

Abbreviations: SD: standard deviation, SE: standard error, PV: P value (Post hoc test). Data described as mean + SD with range in parenthesis or absolute number of patients

Table 2. P value of post hoc tests of renal parameters within groups and between the groups (Tukey HSD)

Dependent variable	Control group <45 yrs	>45 yrs	<45 years group Control	>45 yrs	>45 years group Control	<45 yrs
HbA$_1$C	0.000	0.000	0.000	0.948	0.000	0.948
Urine creatinine	0.000	0.000	0.000	0.822	0.000	0.822
Serum Creatinine	0.034	0.074	0.034	0.000	0.074	0.000
ACR	0.008	0.420	0.008	0.186	0.420	0.186
e-GFR	0.000	0.012	0.000	0.000	0.012	0.000
Calcium	0.048	0.146	0.048	0.871	0.146	0.871
BUN	0.751	0.040	0.751	0.197	0.040	0.197

Table 3. Post hoc test (P value) between study groups of KIM1 and NGAL gene (Tukey HSD)

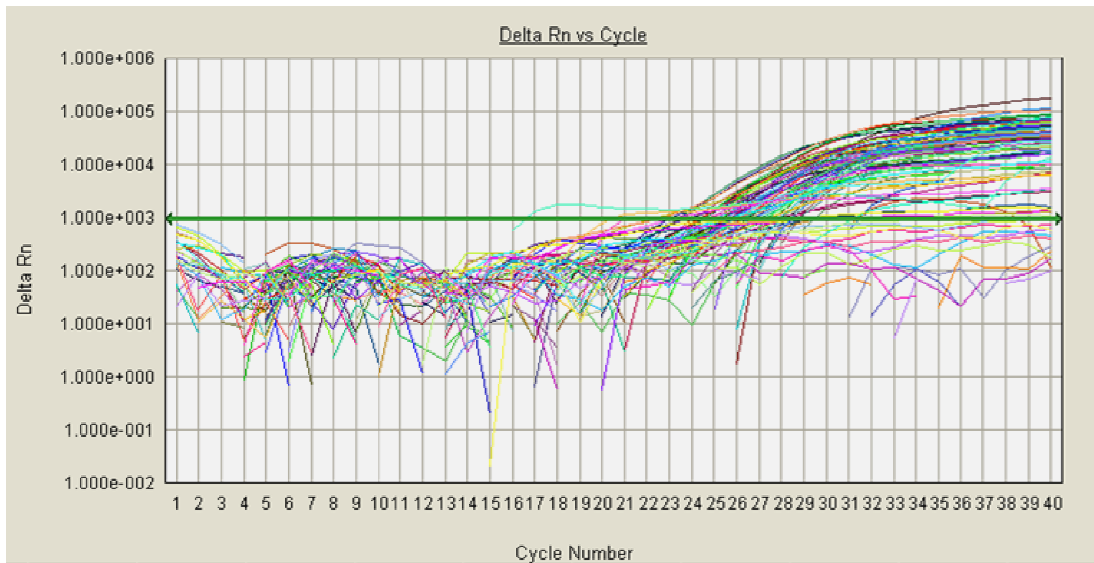| Dependent variable	Control group <45 yrs	>45 yrs	<45 years group Control	>45 yrs	>45 years group Control	<45 yrs
CT OF KIM-1	0.000	0.000	0.000	0.000	0.000	0.000
CTOF NGAL	0.000	0.000	0.000	0.000	0.000	0.000

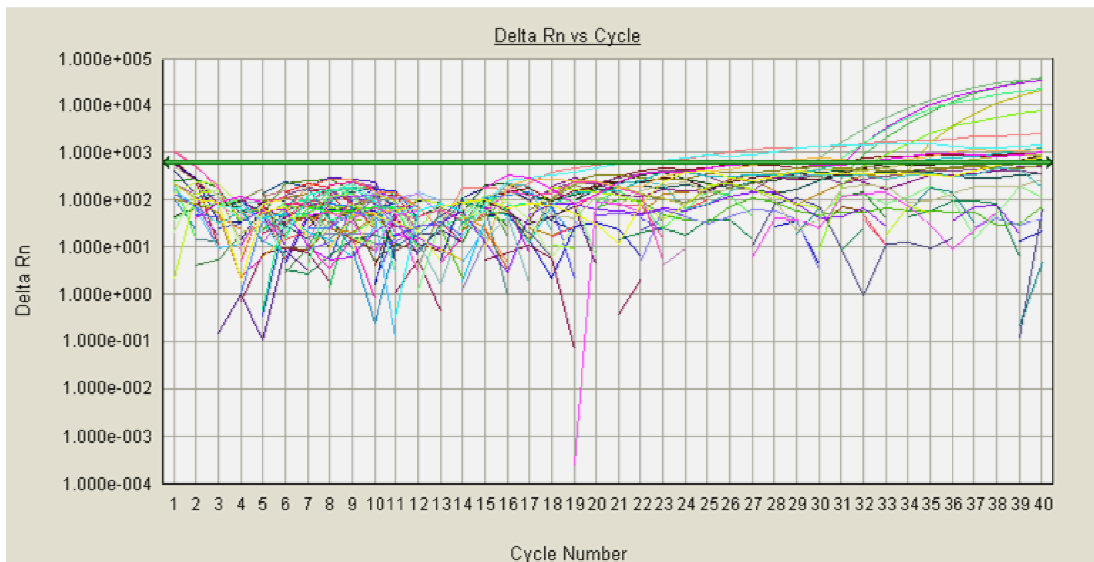**Fig. 1. Real time PCR quantification of KIM-1 plot**

Fig. 2. Real time PCR quantification of NGAL plot

published by Nielsen SE, et al. [30] states that urine-NGAL and urine-KIM1 (u-KIM1) are elevated in Type 1 diabetic patients, with or without albuminuria, indicating tubular damage at an early stage. Study done by Buket Kin Tekce, et al. [31] indicates urinary KIM-1 levels predict renal injury secondary to diabetic nephropathy in early period independent of albuminuria, because urinary KIM-1 was elevated despite normal urinary albumin excretion in the normoalbuminuria subgroup also. From this study it was concluded that urinary KIM-1 has great importance even in normoalbuminuria subjects suffering with T2DM.

Study conducted by Venkata S. Sabbisetti et al. [32] identified blood KIM1 as a marker of kidney injury in humans, where KIM1 levels are significantly elevated in AKI and CKD which predict progression of renal disease in a T1DM cohort study. Literature search found very few study published in T2DM subject on blood sample, so outcome of Venkata S. Sabbisetti study may be useful in T2DM, if tested on

large population. This biomarker may proven potential utility as a sensitive and specific diagnostic and prognostic value for kidney injury. Results of KIM1 in our study are supported by observations of Venkata S. Sabbisetti, et al. study.

The study published by Mahfouz MH, et al. [33] evaluated Neutrophil gelatinase-associated lipocalin (NGAL) and retinol-binding protein 4 (RBP4) in blood for early detection of DN in T2DM patients. In their study they found elevated NGAL and RBP4 levels and both markers were found to correlate positively with duration of diabetes [34]. In our study results of NGAL expressions showed significant p-value (p<0.000) in both the study groups.

5. CONCLUSION

It was concluded that early detection of renal injury in T2DM patients with routine biochemical parameters create dilemma. Outcome of this study evaluated with gene expressions (KIM-1 and NGAL), may help in prediction of risk of DN. Early measurement of KIM-1 and NGAL may prevent ESRD. Further extensive research on large number of subjects with population diversity has been recommended.

CONSENT

All authors declare that 'written informed consent was obtained from patients for publication of outcome of this study' copy of written consent may retrieve from us, if required.

ETHICAL APPROVAL

All authors are here by declared that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Bethesda MD. United States renal data system. USRDS 2007 Annual Data Report: National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, U.S. Department of Health and Human Services; 2007.

2. Barry M Brenner, Mark E Cooper, Dick de Zeeuw, William F Keane, William E Mitch, Hans-HenrikParving, Giuseppe Remuzzi, Steven M Snapinn, Zhonxin Zhang, Shahnaz Shahinfar. Effects of Losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. N Engl J Med. 2001;345:861-869.

3. Ito H, Fujita H, Takahashi T. Diagnostic biomarkers of diabetic nephropathy. Expert Opin Med Diagn. 2008;2(2):161-9.

4. InterAct Consortium, Scott RA, Langenberg C, Sharp SJ, Franks PW, Rolandsson O, et al. The link between family history and risk of type 2 diabetes is not explained by anthropometric, lifestyle or genetic risk factors: The EPIC-InterAct study. Diabetologia. 2013;56(1):60-9.

5. Zelmanovitz T, Gross JL, Oliveira J, De azevedo MJ. Proteinuria is still useful for the screening and diagnosis of overt diabetic nephropathy. Diabetes Care. 1998;21(7):1076-9.

6. Fioretto P, Mauer M, Brocco E, et al. Patterns of renal injury in NIDDM patients with microalbuminuria. Diabetologia. 1996; 39(12):1569-76.

7. Ruggenenti P, Remuzzi G. Nephropathy of type-2 diabetes mellitus. J Am Soc Nephrol. 1998;9(11):2157-69.

8. Silveiro SP, Da Costa LA, Beck MO, Gross JL. Urinary albumin excretion rate and glomerular filtration rate in single-kidney type 2 diabetic patients. Diabetes Care. 1998;21(9):1521-4.

9. Perkins BA, Ficociello LH, Silva KH, Finkelstein DM, Warram JH, Krolewski AS. Regression of microalbuminuria in type 1 diabetes. N Engl J Med. 2003;348(23):2285-93.

10. Caramori ML, Fioretto P, Mauer M. The need for early predictors of diabetic nephropathy risk: Is albumin excretion rate sufficient? Diabetes. 2000;49(9):1399-408.

11. Ichimura T, Bonventre JV, Bailly V, et al. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. J. Biol. Chem. 1998;273:4135-42.

12. Wolf MW, Boldt J. Kidney specific proteins: Markers for detection of renal dysfunction after cardiac surgery? Clin. Res. Cardiol. Suppl. 2007;2:S103–7.

13. Myers BD, Chui F, Hilberman M, Michaels AS. Transtubular leakage of glomerular filtrate in human acute renal failure. Am J Physiol. 1979;237:F319–F325.

14. Sutton TA. Alteration of microvascular permeabilityin acute kidney injury. Microvasc Res. 2009;77:4–7.

15. Coles M, Diercks T, Muehlenweg B, Bartsch S, Zölzer V, Tschesche H, et al. The solution structure and dynamics of human neutrophil gelatinase-associated lipocalin. J Mol Biol. 1999;289(1):139-57.

16. Devarajan P. Neutrophil gelatinase-associated lipocalin — an emerging troponin for kidney injury. Nephrol Dial Transplant. 2008;23(12):3737-43.

17. Bachorzewska-Gajewska H, Malyszko J, Sitniewska E, Malyszko JS, Poniatowski B, Pawlak K, Dobrzycki S. NGAL (neutrophil gelatinase-associated lipocalin) and cystatin C: Are they good predictors of contrast nephropathy after percutaneous coronary interventions in patients with stable angina and normal serum creatinine? Int J Cardiol. 2008;127(2):290-1.

18. Tuladhar SM, Püntmann VO, Soni M, Punjabi PP, Bogle RG. Rapid detection of acute kidney injury by plasma and urinary neutrophil gelatinase-associated lipocalin after cardiopulmonary bypass. J Cardiovasc Pharmacol. 2009;53:261-6.

19. Bennett M, Dent CL, Ma Q, Dastrala S, Grenier F, Workman R, et al. Urine NGAL predicts severity of acute kidney injury after cardiac surgery: A prospective study. Clin J Am Soc Nephrol. 2008;3:665-673.

20. Xin C, Yulong X, Yu C, Changchun C, Feng Z, Xinwei M. Urine neutrophil gelatinase-associated lipocalin and interleukin-18 predict acute kidney injury after cardiac surgery. Ren Fail. 2008;30: 904-13.

21. Liangos O, Tighiouart H, Perianayagam MC, Kolyada A, Han WK, Wald R, et al. Comparative analysis of urinary biomarkers for early detection of acute kidney injury following cardiopulmonary bypass. Biomarkers. 2009;14:423-31.

22. Bolignano D, Lacquaniti A, Coppolino G, Campo S, Arena A, Buemi M. Neutrophil gelatinase-associated lipocalin reflects the severity of renal impairment in subjects affected by chronic kidney disease. Kidney Blood Press Res. 2008;31(4):255-8.

23. Bolignano D, Coppolino G, Campo S, Aloisi C, Nicocia G, Frisina N, et al. Urinary neutrophil gelatinase-associated lipocalin (NGAL) is associated with severity of renal disease in proteinuric patients. Nephrol Dial Transplant. 2008;23(1):414-6.

24. Xu SY, Carlson M, Engström A, Garcia R, Peterson CG, Venge P. Purification and characterization of a human neutrophil lipocalin (HNL) from the secondary granules of human neutrophils. Scand J Clin Lab Invest. 1994;54(5):365–376.

25. Kjeldsen L, Johnsen AH, Sengeløv H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem. 1993;286:10425–10432.

26. Nielsen SE, Andersen S, Zdunek D, Hess G, Parving HH, Rossing P. Tubular markers do not predict the decline in glomerular filtration rate in type 1 diabetic patients with overt nephropathy. Kidney Int. 2011;79:1113–1118.

27. Waanders F, Waanders F, van Timmeren MM, Stegeman CA, Bakker SJ, van Goor H. (January 2009). Kidney injury molecule-1 in renal disease. J Pathol. 2010;220(1): 7-16.

28. Markus LA, Axcl K, Karoline VW, Oleg T, Christoph R, Alexandra S, et al. Early urinary and plasma biomarkers for experimental diabetic nephropathy. Clin. Lab. 2012;58:659-671.

29. Vaidya VS, Ramirez V, Ichimura T, Bobadilla NA, Bonventre JV. Urinary kidney injury molecule-1: A sensitive quantitative biomarker for early detection of kidney tubular injury. Am J Physiol Renal Physiol. 2006;290:F517-29.

30. Nielsen SE, Sugaya T, Hovind P, Baba T, Parving HH, Rossing P. Urinary liver-type fatty acid-binding protein predicts progression to nephropathy in type 1 diabetic patients. Diabetes Care. 2010;33: 1320–1324.

31. Buket Kin Tekce, Hikmet Tekce, Gulali Aktas, Mustafa Sit. Evaluation of the urinary kidney injury molecule-1 levels in patients with diabetic nephropathy. Clin Invest Med. 2014;37(6):E377-E383.

32. Venkata S Sabbisetti, Sushrut S Waikar, Daniel J Antoine, Adam Smiles, et al. Blood kidney injury molecule 1 is a

biomarker of acute and chronic kidney injury and predicts progression to ESRD in type I diabetes. J Am Soc Nephrol. 2014;25(10):2177–2186.

33. Mahfouz MH, Assiri AM, Mukhtar MH. Assessment of Neutrophil gelatinase-associated lipocalin (NGAL) and Retinol-binding protein 4 (RBP4) in type 2 diabetic patients with nephropathy. Biomark Insights. 2016;11:31-40.

34. Schmidt-Ott KM, Mori K, Li JY, et al. Dual action of neutrophil gelatinase-associated lipocalin. J Am Soc Nephrol. 2007;18:407–413.