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The Differences of N–Acetyl–β–Glucosaminidase and β2 Microglobulin levels in Patients with and without Early Diabetic Nephropathy

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ABSTRACT

Diabetic Nephropathy (DN) becomes the most serious microvascular complication, which is characterized by the presence of persistent albuminuria. N–acetyl–β–glucosaminidase is a dominant lysosomal enzyme in renal tubular epithelial cells. β2 microglobulin is a low molecular weight protein produced by Major Histocompatibility Complex Class 1 (MHC-1) expressed in all nucleated cells. Both N–acetyl–β–glucosaminidase and β2 microglobulin could be a new useful marker for early detection of DN. Analytic observational study with a cross-sectional approach was performed from May to July 2019 involving 27 non-diabetic patients (K1), 27 diabetic patients without DN (K2), and 27 diabetic patients with early DN (K3) at the Clinical Pathology Department of the Faculty of Medicine, Diponegoro University and Diabetic Clinic. Data presented in this study were age, gender, fasting blood glucose, blood pressure, and urine albumin creatinine ratio. N–acetyl–β–glucosaminidase levels between groups were analyzed using ANOVA, β2 microglobulin levels between groups were analyzed using Kruskal-Wallis test, $p<0.05$ was considered significant. This study showed that there were significant differences in NAG level and β2M level among three groups consisting of non-DM patients, DM patients with and without early DN. It was suggested that both parameters can be used as alternative markers in diabetes mellitus with early DN.

Keywords: Diabetic nephropathy, N-acetyl-β-glucosaminidase, β2 microglobulin, urine albumin creatinine ratio

INTRODUCTION

The prevalence of diabetes is increasing every year all over the world. Based on the data by the International of Diabetic Federation (IDF), the global prevalence rate of people with Diabetes Mellitus (DM) in 2014 was 8.3%. Indonesia was the 7th most populated country with DM in the world, which had 8.5 million people with DM.[1] The diabetic proportion based on Riset Kesehatan Dasar (RISKESDAS) in Indonesia was 10.9% with most of them at the age of 35-54 years old.[2]

Diabetic Nephropathy (DN) is one of the serious microvascular complications of diabetes. It is the main cause of chronic kidney disease to End-Stage Renal Disease (ESRD).[3] Data of RISKESDAS in 2018 showed that the prevalence of the Indonesian population who had chronic kidney disease was 0.38%.[2] Diabetic nephropathy is a clinical syndrome characterized by persistent urine albumin (> 300 mg/g or > 200 µ/min), clinically at a minimum of two tests in a period of 3 to 6 months, followed by increased blood pressure and a decreased Glomerular Filtration Rate (GFR). Urine albumin in diabetic patients is considered as a sensitive and specific marker of kidney damage to diagnose and give the treatment of early diabetic nephropathy.[4] Diabetic nephropathy is diagnosed based on normal albumin level (<30 mg/g creatinine) and microalbuminuria (30-300 mg/g creatinine).[4,5]

Urine albumin is a parameter of endothelial dysfunction in which endothelial defense functions are lost and leakage of protein or albumin occurs. It can be predicted on early DN by measuring Albumin Creatinine Ratio (ACR).[5,6] However, ACR has several limitations such as low sensitivity and large variability. In some cases, a decrease in GFR can occur with normal urinary albumin excretion. The high sensitivity (81.8%) and specificity (96.8%) of microalbuminuria is the reason why it is necessary to perform further studies by using a marker with higher sensitivity.[7,8] Several markers of tubular and glomerular damage have been investigated for early DN markers.

N-acetyl-β-glucosaminidase (NAG) is a lysosomal enzyme found in proximal tubular epithelial cells and has a relatively high molecular weight. Therefore, it is not filtered through the glomerulus and is only

released into the urine after tubular damage. N-acetyl-β-glucosaminidase has a higher diagnostic level compared to albumin excretion in diabetic patients, even NAG level can increase in normoalbuminuria.[7,8] Sheira *et al*. suggested that NAG could be used as a marker of early DN because urine NAG increased in tubulointerstitial damage.[7]

β2 microglobulin is a low molecular weight protein produced by cells expressing Major Histocompatibility Complex Class 1 (MHC-1) and found in all nucleated cells. Because it is almost entirely filtered by the glomerulus, it can be used to determine the glomerular filtration rate.[8,9] The precision and accuracy of GFR estimation with creatinine were dissatisfactory because it is influenced by an external factor such as the variation of muscle mass. β2 microglobulin can be used as an additional marker to measure LFG.[10,11] In addition, Abdullah *et al*. suggested that it also could be used as a marker of early DN.[10]

The researchers conducted a study to investigate the difference of NAG and β2M levels in non-DM patients, type 2 DM patients without diabetic nephropathy, and type 2 DM patients with early diabetic nephropathy. This study aimed to gain information on alternative parameters as a marker of early DN.

METHODS

This was an observational analytic study with a cross-sectional design conducted between May to July 2019 by observing type 2 DM patients in Kagok Community Health Center, Semarang, and non-DM patients in the Department of Clinical Pathology, Medical Faculty of Diponegoro University. N–acetyl–β–glucosaminidase and β2 microglobulin levels were measured using Enzyme-Linked Immunosorbent Assay (ELISA) method in GAKI Laboratory Medical Faculty Diponegoro University, whereas urine albumin (ACR) was tested on Sarana Medika Laboratory Semarang. Samples were taken consecutively. The research subjects were type 2 DM patients with and without diabetic nephropathy taken from Kagok Community Health Center, Semarang and non-DM patients taken from the Department of Clinical Pathology, Medical Faculty of Diponegoro University who were willing to participate in this study, had signed informed consent and met the inclusion and exclusion criteria. The characteristic of subjects (age, gender, blood pressure, fasting blood glucose, and urine albumin creatinine ratio) was collected from the patient's medical record.

The sample size in this study was 81 patients. Data were presented in median (minimum-maximum). They were divided into 3 groups; group I: 27 non-diabetic (healthy persons), group II: 27 DM patients without nephropathy, and group III: 27 DM patients with early DN. Inclusion criteria of non-DM patients in this study were males and females, aged 30-55 years old, fasting blood glucose < 110 mg/dL, normal body temperature, and normal blood pressure. Inclusion criteria of DM patients without nephropathy were males and females, aged 30-55 years old, fasting blood glucose >110 mg/dL, normal body temperature and blood pressure (≤ 120/80 mmHg) with normoalbuminuria (ACR ≤ 30 mg/g). Inclusion criteria of DM patients with early DN were males and females, aged 30-55 years old, fasting blood glucose >110 mg/dL, normal body temperature, and normal blood pressure (≤ 120/80 mmHg) with microalbuminuria (ACR 30–300 mg/g). Exclusion criteria for three groups were patients with liver diseases, cardiovascular diseases, cancer, or smoking habit.

Data were processed using SPSS 15. Because the sample size was less than 50, the Shapiro-Wilk test was used to determine the data normality. Multivariate analysis was performed on each variable to determine sample characteristics. N-acetyl-β-glucosaminidase levels were firstly transformed due to abnormal distribution. However, because it remained abnormally distributed in spite of the transformation, the Kruskal-Wallis test was used. Contrastingly, due to normal distribution, β2 microglobulin data were analyzed using the oneway ANOVA test. Both the statistical tests showed significant results, so the analysis was continued with the Post-Hoc test. $p < 0.05$ was significant.

The research has obtained ethical clearance from the Health Research Ethics Commission, Faculty of Medicine, Diponegoro University, Semarang with number 235/EC/FK-UNDIP/VI/2019.

RESULTS AND DISCUSSIONS

From the total of 90 subjects who took part in the study, 81 subjects met inclusion and exclusion criteria, consisting of 27 non-DM patients, 27 type 2 DM patients without nephropathy, and 27 type 2 DM patients with early diabetic nephropathy. However, 9 subjects dropped out because 3 subjects had a history of heart disease, 1 subject had a history of malignancy, 5 subjects had high blood pressure.

The characteristics of subjects were presented in the in mean±SD and median (min;max) (Table 1).

Table 1. General characteristics of research subjects

Variable	Group					
	Non-DM		DM without Nephropathy		DM with Early DN	
	Mean±SD	(n)%	Mean±SD	(n)%	Mean±SD	(n)%
Gender						
Male		9(33.3)		7(25.9)		9(33.3)
Female		18(66.7)		20(74.1)		18(66.7)
Age (year)	34.33±5.44		50.41±3.04		50.89±2.87	
Fasting blood glucose (mg/dL)	94.85±6.19		244,48±106,45		241,81±95.54	
Systolic (mmHg)	118,52±4.56		120.19±5.46		118,33±4.16	
Diastolic (mmHg)	78.15±3.96		80.00±5.55		78.52±4.56	
ACR (mg/g)	6.65±3.42		13.96±6.12		89.56±55.61	

Table 2. Differences in NAG and β2M levels in non-DM, DM patients without nephropathy, and DM patients with early diabetic nephropathy

Group	N	NAG Median (min-max) ng/mL	p	β2M Mean ±SD ng/mL	p
Non-DM	27	1.91 (0.56-4.38)	0.001†*	2009.52±336.77	0.017§*
DM without nephropathy	27	4.09 (0.89-13.5)		2190.85±212.07	
Non-DM	27	1.91(0.56-4.38)	<0.001†*	2009.52±336.77	<0.001§*
DM with early DN	27	6.88 (1.49–24.85)		2773.45±257.17	
DM without nephropathy	27	4.09 (0.89-13.5)	0.003†*	2190.85±212.07	<0.001§*
DM with early DN	27	6.88 (1.49–24.85)		2773.45±257.17	

NAG: N–acetyl–β–glucosaminidase, β2M: β2 microglobulin,*Significant (p<0.05); † Post-Hoc Mann-Whitney. § Post-Hoc LSD

Table 1 showed that most of the subjects were female. The mean age of DM patients was higher than non-DM patients. The mean age of DM patients without nephropathy and early diabetic nephropathy was similar, which was 50 years old. Urine albumin levels of non-DM patients had a normal median (6.65 mg/g). Type 2 DM patients without nephropathy were included in the normoalbuminuria group because their median urine albumin level was 13.96 mg/g, while type 2 DM patients with early DN was included in the microalbuminuria group (30-300 mg/gr creatinine).

Due to the abnormal distribution of NAG levels in the three groups, data were then presented in the median (min-max). Contrastingly, due to the abnormal distribution of β2M levels in the three groups, data were then presented in mean±SD. The analysis result of NAG and β2M levels in the three groups can be seen in Table 2.

The results of the statistical test among all study groups were shown in Table 2. There were significant differences in N–acetyl–β–glucosaminidase levels between K1 and K2 ((p=0.01), K1 (non-DM and DM without nephropathy), and K3 ((p =< 0.01), K2 and K3 (p=0.03). β2 microglobulin between K1 and K2 (p=0.02), K1 and K3 (p =< 0.01), K2 and K3 (p< 0.01). N-acetyl-β-glucosaminidase and β2 microglobulin levels were higher in DM patients compared to non-DM patients and increased higher in DM patients with early DN compared to non-DM patients and DM patients without nephropathy.

The percentage of female patients was greater than males in this study. The cause of this finding remains debatable among experts, but it was suspected that type 2 DM occurred in most females at the age of nearly half a century. This was supported by a study by Alotaibi *et al*., which found four studies reported significantly higher prevalence

rates for type 2 DM patients in males than in females; one regional study from the Eastern province and two nationwide studies, conducted between 2004 and 2005 and between 2007 and 2009 reported significantly higher prevalence rates for type 2 DM patients among females than males.[12]

Non-DM subjects were younger compared to DM subjects with a mean age of 33 years. The mean age of DM patients with and without DN was almost similar, around 50 years. These results were similar to those in a study by Retnakaran *et al*., which found that individuals aged ≥ 45 years had a greater risk of developing DM compared with individuals aged <45 years, and in accordance with several epidemiological studies, which suggested that the susceptibility level of type 2 DM was proportional with increasing age.[13] According to RISKESDAS in 2018, the incidence of type DM was greatest at the age 35-54 years (10.9%).[2]

Albumin creatinine ratio in non-DM and type 2 DM subjects without nephropathy were normal (ACR levels < 30 mg/g) of 5 and 12, respectively. It is an early predictor of DN complications assessed by measuring ACR.[5] DM patients with early DN was included in the microalbuminuria group (ACR levels of 30-300 mg/g). Urine albumin in DM patients is considered the best predictor of kidney damage in assisting early diagnosis of DN. It is a parameter of endothelial dysfunction in which endothelial defense functions are lost and leakage of protein or albumin occurs.[5,14]

The median blood glucose level in non-DM patients was within the normal range of ≤ 126 mg/dL. The median blood glucose level in type 2 DM patients without nephropathy and with early DN was ≥ 126 mg/dL. The mean fasting blood glucose of type 2 DM patients without nephropathy was higher than type 2 DM with DN patients. This might be influenced by the duration of fasting time, oral antidiabetic drug doses, and medication compliance.

The statistical test result of NAG levels in non-DM patients was significantly lower than type 2 DM patients without nephropathy (p=0.001). According to a study by Hong *et al*., NAG is a lysosomal enzyme, which is normally excreted in small amounts in urine through physiological exocytosis in proximal tubular cells, which was significantly higher in type 2 DM patients compared with healthy subjects.[8,15] Kaufmann *et al*. also found that there was a positive relationship between urine NAG and creatinine clearance in type 2 DM patients. The increase in urine NAG was proportional to a decreased kidney function in the early stages of DN development.[16]

The increase in urine NAG levels was due to the increased activity of proximal tubular cell lysosomes because of injury of tubular cells.[15,17] This study was in accordance with previous studies, including a study by Siddiqui *et al*., which reported that Cystatin C, NGAL, and NAG biomarkers were significantly higher in Diabetic kidney disease.[18]

The NAG levels in non-DM patients were significantly lower than type 2 DM patients with early DN (p<0.001). Increased level of NAG portrayed the level of kidney tubules damage. Sheira *et al*. stated that an increase in NAG observed in three groups of patients with albuminuria was directly proportional to the severity of kidney disorders.[7]

Microalbuminuria is a clinical marker of diabetic nephropathy, but it is clinical manifestation can only be found in stage three of the five stages of DN development.[8,17] Lysosomal enzyme from the tubular cell injury is secreted into the urine during the development of diabetic nephropathy. The activity of lysosomal enzymes shows an increase in the development of DN earlier than microalbuminuria.[7,17]

The NAG level of type 2 DM patients without nephropathy was significantly lower than type 2 DM patients with early DN (p=0.003). Increased NAG through the process of exocytosis by proximal tubular cells triggered proteinuria. This showed the relationship between urine NAG level and the severity of proteinuria.[18]

According to a previous study, the detection of microalbuminuria in DM patients showed glomerular involvement in early kidney damage.[15] Recent studies showed tubular involvement in DN complications, as demonstrated by the presence of renal tubular protein and enzyme in urine. Tubular involvement preceded the glomerular because many of this protein and enzyme tubular could be detected even before microalbuminuria occurred. At the beginning of glomerular damage, increased albumin leakage could be absorbed by the tubules if its function was still normal that albuminuria wouldn't occur.[14,19]

The mean of β2M levels in non-DM patients was lower than type 2 DM patients without nephropathy. The β2M level increased in accordance with the degree of renal dysfunction.[9] The median of β2M levels in non-DM patients and type 2 DM patients without nephropathy was 2085,9 and 2161,5, respectively. The normal median of β2M levels indicated the absence of kidney dysfunction. The β2M measurement was used in this study to assess kidney function.[10] Many studies had analyzed the ability of β2M to assess renal function and its levels

were found superior to creatinine serum because the decreasing of GFR just starts in stage 3 of diabetic nephropathy.[11]

There were significant differences in β2M levels in non-DM patients and type 2 DM patients without nephropathy (p=0.017). This might be due to increased serum β2M level in inflammatory conditions. In type 2 DM patients, chronic hyperglycemia-induced the formation of AGE. Increased expression of AGE in the RAGE receptor in endothelial cells triggered oxidative stress and induced the production of proinflammatory cytokines.[10,11] Valdet *et al*. stated that hyperglycemia significantly increased inflammatory markers such as TNF-α and IL-6, which played a role in the pathogenesis of type 2 DM and its complications such as atherosclerosis and neuropathy.[20] Vaia *et al*. also stated that hyperglycemia played a significant role in the production of inflammatory cytokines.[21]

The mean of β2M level in non-DM patients was significantly lower than type 2 DM patients with early DN (p<0.001). Serum β2M can be used as a marker of chronic kidney disease associated with kidney dysfunction, especially in glomerular disorders. Measurement of the GFR index was considered less sensitive in the early stages of the disease because it could be normal.[20] Colombo *et al*. reported that β2M concentrations increased significantly in the normoalbuminuria and microalbuminuria groups compared with controls.[22] Many studies identified the potential of β2M as a marker for diabetic renal disease and end-stage renal disease.[20,22]

The mean of β2M levels in type 2 DM patients without nephropathy was significantly lower than type 2 DM patients with early DN (p <0.001). DM patients with early DN had a mean β2M above the normal value of 1.100-2.600 ng/mL. The increase of β2M occurred in patients with renal dysfunction.

This study was in accordance with several previous studies, which reported that the microalbuminuria group had an increase in β2M compared to the normoalbuminuria group. Colombo *et al*. stated that β2M level was higher in DM patients with microalbuminuria compared to patients without microalbuminuria and the control group.[22]

This study did not consider the duration of DM, which might influence NAG and β2M. Another study, which uses a larger sample size and considers other risk factors of diabetic nephropathy, which may affect study variables should be performed. A diagnostic study was also considered necessary to be conducted.

CONCLUSIONS AND SUGGESTIONS

This study showed that there were significant differences in NAG and β2M levels in three groups, which each consisted of non-DM patients, DM patients with and without early DN. This research suggested that both parameters can be useful to be alternative markers in early DN.

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