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SERUM *PENTOSIDINE* ASSAY AMONG DIABETIC NEPHROPATHY PATIENTS IN SOUTH-SOUTH NIGERIA

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ABSTRACT

Introduction: AGEs have been implicated in the causation of chronic renal failure, long term dialysis-related amyloidosis and atherosclerosis. One of such is pentosidine on which there is a paucity of literature on its biomarker properties for nephropathy in sub-saharan Africa. This study aimed to assess serum Pentosidine levels in diabetic nephropathy. **Method:** A hospital based cross sectional study involving 80 DN cases being managed in the University of Benin Teaching Hospital. The controls were 80 non diabetic persons from the University of Benin Teaching Hospital. The cases were selected using systematic sampling method and data collected using structured questionnaire after obtaining informed consent. They were sampled for plasma Pentosidine, Serum Creatinine, Serum Urea, Urinary Albumin and Creatinine. Urinary albumin-to- creatinine ratio and creatinine based glomerular filtration rate were calculated from the results. Data was analyzed using IBM SPSS version 22 and presented in form of tables, charts and graphs. **Results:** Of the total of 160 patients, 80 were DN cases and 80 were non diabetic controls. The study showed a significant difference between the mean levels of serum creatinine and urea, urinary creatinine and albumin, eGFR, and serum pentosidine levels between the cases and controls. The diagnostic accuracy of pentosidine assay in DN was 96.86% compared to 95.62% of UACR. **Conclusion:** Serum pentosidine assay was shown to have a high diagnostic accuracy in diagnosing diabetic nephropathy.

KEYWORDS: Pentosidine, Estimated Glomerular Filtration Rate(Egfr), Diabetic Nephropathy.

BACKGROUND

Advanced Glycation End Products (AGEs) have a central role in the pathogenesis of the vascular Complications of diabetes.[1] AGEs are actually a complex group of compounds formed via a non enzymatic reaction between reducing sugars and amine residues on proteins, lipids or nucleic acids. The pathogenesis includes the non-enzymatic reaction of glucose with proteins leading to the formation of Schiff base and ultimately Amadori products.[2] Amadori products would further lead to formation of advanced glycated end products (AGEs).[3] Development of DN occurs from chronic hyperglycemia which leads to oxidation of proteins with formation and consequent accumulation of AGEs.[4] Several theories have been postulated as to how macro and micro-vascular diseases in diabetes occur from chronic hyperglycemia. And one of these, is the theory of AGEs.[5] Proof as to how diabetic micro-vascular complications develop from AGEs have been reported from in vitro and in vivo research works that explored the role of AGEs in different pathologies. These studies have been corroborated by other studies that demonstrated substantial improvements of signs and symptoms by anti-AGE agents on diabetic complications.

Hyperglycaemia up regulates intracellular formation of AGEs, with the most abundant AGE present being carboxy methyl lysine (CML) the levels of which have been suggested to be associated with incidence of diabetic complications.[6]

Pentosidine is one of the other well-defined AGE products to date. AGEs exert their effects via binding to receptors for AGE (RAGE) which are expressed by several cells. AGE-RAGE interaction has clearly been demonstrated to be involved in the development of microvascular complications.[6]

Diabetic nephropathy (DN) is a clinical syndrome in a background diabetes mellitus (DM) that is characterized by a progressive and persistent albuminuria confirmed on at least two occasions three to six months apart or characterized by a non-reversible decline in glomerular filtration rate(GFR), intra-renal hypertension and elevated arterial blood pressure.[7,8] It is a progressive kidney disease caused by angiopathy of capillaries in the glomeruli of the kidney.[9] It causes premature mortality, end-stage renal disease (ESRD) which lead to renal replacement therapy, cardiovascular diseases, and expensive health-care burden.[10] It is a major complication in persons with DM.[9]

Thus the need for early diagnosis of DN cannot be overemphasized. Presently the most widespread means of diagnosis is the use of urinary albumin-creatinine ratio. The use of urinary albuminuria in the determination and monitoring of DN has limitations in non-proteinuric DN. Estimation of urinary albumin-to-creatinine ratio using urinary albumin and creatinine also has limitations as urinary creatinine concentration may be elevated by exercise within 24 hours, infection, fever, congestive cardiac failure, menstruation, marked hypertension and marked hyperglycaemia independent of kidney damage. There is need for newer frontiers to be explored, one of which is the use of advanced glycated end products (AGEs) as predictors of DN. As the intricacies of biochemical mechanisms underlying the pathogenesis of DN are being unraveled, a single biomarker for risk stratification would be ideal in personalized and predictive medical practice.[11]

One of the best chemically characterized AGE is Pentosidine, which acts as a marker for the production and deposition in tissues of AGEs in persons with DM.[13] Pentosidine has been characterized and has a well-known structure among AGEs and also implicated in the causation of chronic renal failure, long term dialysis-related amyloidosis and atherosclerosis.[14] Studies on it as a biomarker of Diabetic nephropathy are scarce in sub-saharan Africa.

Pentosidine is a fluorescent cross-linking molecule.[14] Cross-linking AGEs are responsible for an increasing proportion of insoluble extracellular matrix and thickening of tissue and also as increasing mechanical stiffness with loss of elasticity.[15] Pentosidine is implicated in nephrotoxicity via alteration of proteins tertiary structures through cross-linking, altering enzymatic activity and impairing receptor recognition.[16] It has been reported that Pentosidine is present in renal basement membranes of glomeruli.[17] This study aims to evaluate the correlation between serum pentosidine levels and severity of DN. It assessed serum *Pentosidine* as a biomarker in diabetic nephropathy patients selected with *Creatinine* based estimated glomerular filtration rate. Specifically, it measured serum *pentosidine* levels in patients with diabetic nephropathy and in non-diabetic control individuals; Compare serum *Pentosidine* with Creatinine based eGFR in selecting cases for the study.

MATERIALS AND METHODS

The study was conducted in the Department of Chemical Pathology of the University of Port Harcourt Teaching Hospital. River State, Southsouth Nigeria. The state has a total of 23 Local Government Areas (LGAs) with a population density of 470 square kilometers and total population of 5, 198, 716 (2006 census). It is bounded in the south by the Atlantic Ocean, north by Imo, Abia and Anambra states, east by Akwa-Ibom state and west by Bayelsa and Delta states. Port Harcourt being the capital of Rivers State is cosmopolitan in nature and habours people of different backgrounds. Ethical approval obtained from the University of Port Harcourt Teaching Hospital. An Informed written consent was equally obtained from the study participants as well as the control subjects.

The study population was made up of adult diabetic nephropathy patients. The cases included both recently diagnosed patients and patients on admission. Controls were non diabetic persons from the University of Benin Teaching Hospital. Eighty (80) diabetic nephropathy patients (cases) and 80 non diabetic controls were used for this study. The sample size was calculated from the standard formula.[18]

About ten millilitres (10 mls) of venous blood was collected with syringe and needle from the ante-cubital vein of all patients and divided into lithium heparin and plain bottles as whole blood. The contents of the lithium heparin bottles were centrifuged at 2000-3000 revolutions per minutes (rpm) and plasma were then collected into plain 5 ml labeled bottles with Pasteur pipette. The contents of the plain bottles were allowed to clot for 2 hours. This was followed by a 10 minutes centrifugation at 2000-3000 rpm, and sera were then collected into 5ml labeled plain bottles. Samples for serum pentosidine were kept at -70^0C until analysis. The samples were labeled with a unique identification number, and date of collection. All sample collection was done under standard operating procedure. Every batch of sample was processed alongside a control. Access to patient data/information was restricted to the researcher/supervisor and assessors. Serum levels of pentosidine were measured by Enzyme linked immunosorbent Assay (ELISA). ELISA is a highly sensitive and specific heterogenous enzyme immunoassay technique that uses antigen and antibody linked to enzymes for detection of analytes.[19,20] Calculation: The corresponding concentrations of the standards, controls and samples in ng/ml were calculated from their absorbances using a standard curve generated by the ELISA microplate reader.[19]

Urea: Serum levels of urea was measured by Urease-Berthelot Method.[21] Urea in serum is hydrolysed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot's reaction.[21]

The albumin concentration of urine samples were measured by Turbidimetric Immunoassay.[23] In this method, determination of urinary albumin is based on the principle of agglutination reaction. The test specimen is mixed with the activation buffer (R1) and anti-human antibody solution (R^2) and allowed to react.[22] Presence of albumin in the test specimen forms an insoluble complex producing a turbidity, which is measured at wavelength 340 nm. The resulting turbidity corresponds to the concentration of albumin in the test specimen.

Levels of creatinine were measured by Modified Jaffe's kinetic Reaction[23] Picric acid in an alkaline medium

reacts with creatinine to form an orange coloured complex with the alkaline picrate. Intensity of the colour formed is directly proportional to the amount of creatinine present in the sample. Creatinine + Alkaline Picrate gives an orange coloured complex.[23]

Data obtained during the course of the study were analyzed using statistical package for social sciences (SPSS) version 25. Results were expressed as mean, standard deviation and proportions and presented in tables and charts as appropriate. Categorical variables were compared using chi squared test and fisher's exact. Continuous variables were compared using t test.

Pearson's correlation coefficient was used to compare *Pentosidine* with eGFR. Receiver operating characteristics curve (ROC) analysis was used to determine sensitivity and specificity of each assay. *Pentosidine* assays results were analyzed using receiver operating characteristic (ROC) analysis by SPSS 22; cut-off (CO) values for the test were set according to the diagnostic accuracy. Accordingly, the maximum CO value for *Pentosidine* was set to 17.5690 which was equal to the point where Y is maximum (Y = (sensitivity + specificity)/2). Level of probability was set at $P \leq 0.05$.

RESULTS

A total of 160 cases participated; 80 cases being DN patients and 80 being controls. The findings of the results are as follows

Table 1: Socio-demographic characteristics of Age Sex distribution of study subjects.

	DN n = 80	Control n = 80	P value
Age group			
40 – 49	4 (5.0)	7 (8.8)	
50 – 59	33 (41.2)	44 (55.0)	***0.004****
60 – 69	39 (48.8)	18 (22.5)	
>=70	4 (5.0)	11 (13.8)	
Sex			
Male	42 (52.5)	37 (46.2)	0.429
Female	38 (47.5)	43 (53.8)	
Weight			
≤68.417	7 (8.8)	22 (27.5)	***0.006****
68.418 – 76.238	19 (23.8)	16 (20.0)	
76.239 – 84.058	49 (61.3)	33 (41.3)	
≥84.059	5 (6.3)	9 (11.3)	

Significant

Table 2: Mean values of the cases and controls study subjects.

	DN Mean±SD	Control Mean±SD	t value	P value
Age	59.73±6.43	58.13±8.41	-1.351	0.179
Weight	75.04±9.53	77.43±5.40	-1.958	0.052
Serum Creatinine	4.85±2.67	1.02±0.21	-12.805	< ***0.001****
Serum Urea	113.73±54.5	25.7±7.09	-14.326	***0.001****
Urinary Creatinine	58.86±19.43	129.12±29.01	17.995	< ***0.001****
Urinary Albumin	11.36±3.35	3.13±0.77	-21.424	< ***0.001****
UACR	222.03±103.51	26.05±11.06	-16.840	< ***0.001****
eGFR	25.47±15.19	97.30±14.10	30.998	< ***0.001****

Significant

Thirty nine (48.8%) of the cases were aged 60 – 69 years compared to 44 (55.0%) of the controls aged 50 – 59 years. There was a statistically significant association between the age of the patients and their diagnostic groups (p = 0.004).

Forty two (52.5%) of the cases were males compared to 43 (53.8%) of the controls that were females. This association was however not statistically significant (p = 0.429).

The mean age of the cases was 59.73 ± 6.43 years compared to 58.13 ± 8.41 years of the controls. There were no statistically significant differences between the mean age of the cases and controls (p = 0.179).

The mean weight of the cases was 75.04 ± 9.53 kg compared to 77.43 ± 5.40 kg of the controls. There was also no statistically significant difference between the means (p = 0.052).

The mean value of serum urea among the cases was 113.73 ± 54.5 mg/dl compared to 25.7 ± 7.09 mg/dl among the controls. This difference between the means was statistically significant (p = 0.001).

The mean value for urinary creatinine among the cases was 58.86 ± 19.43 mg/dl compared to 129.12 ± 29.01 mg/dl of the controls. This difference was also statistically significant (p < 0.001).

The mean value for urinary albumin among the cases was 11.37 ± 3.35 mg/dl compared to 3.13 ± 0.77 mg/dl of the controls. This difference was statistically significant (p < 0.001).

The mean value for urinary albumin-to- creatinine ratio among the cases was 222.03 ± 103.51 mg/g compared to 26.05 ± 11.06 mg/g among the controls. This difference was statistically significant (p < 0.001).

The mean value for eGFR among the cases was 25.47 ± 15.19 ml/min compared to 97.30 ± 14.10 ml/min among the controls. There was a statistically significant difference between the means (p < 0.001).

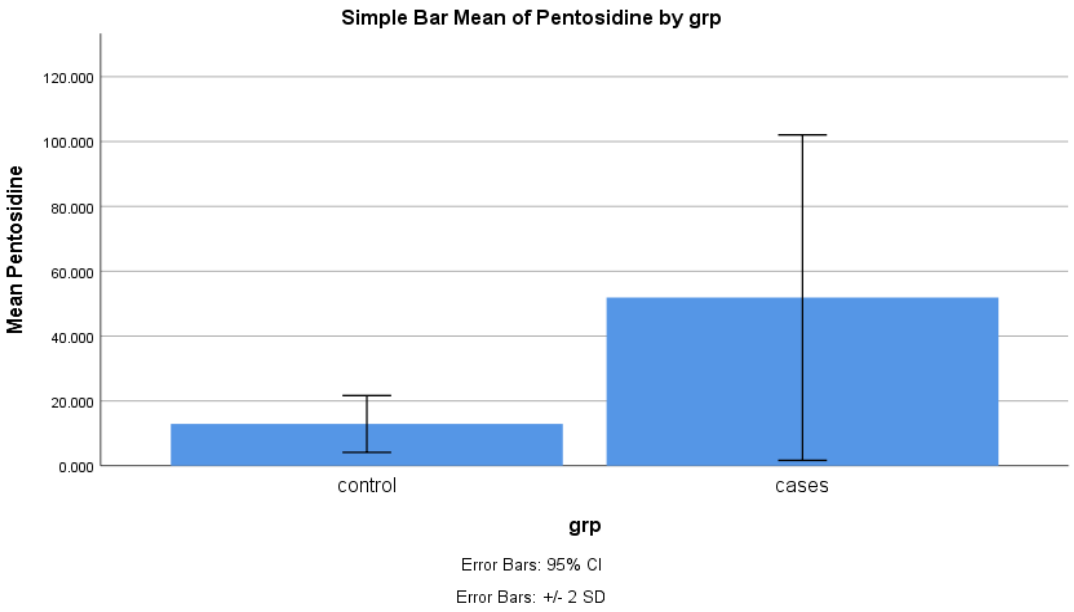


Figure 1: Mean Pentosidene levels between the cases and controls.

The mean pentosidine level among the cases was 51.87 ±25.09 ng/ml compared to 12.90 ±4.38 ng/ml of the controls. This difference was also statistically significant (t = 13.685; p < 0.001).

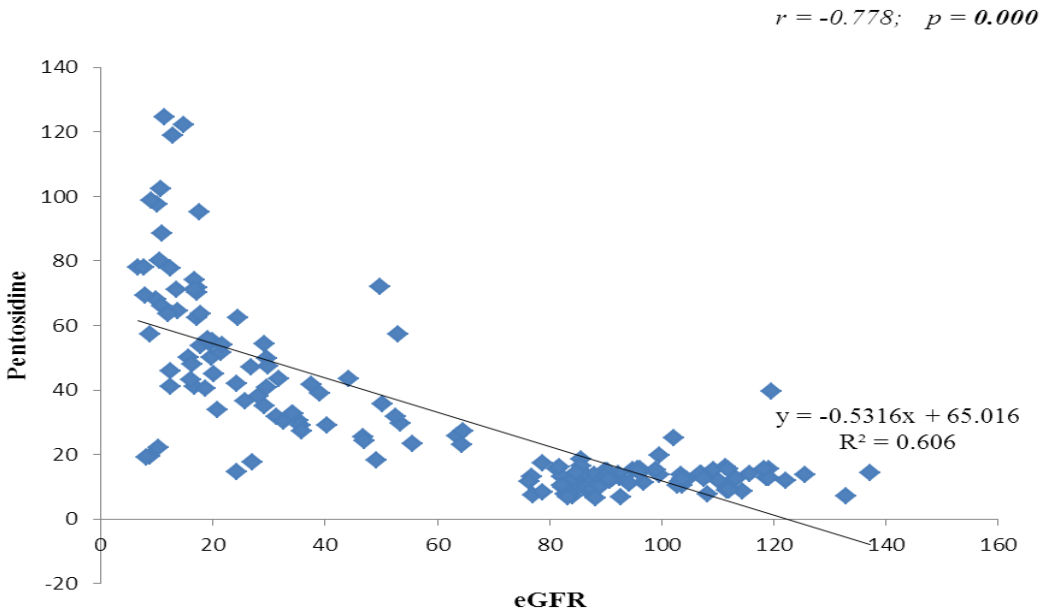
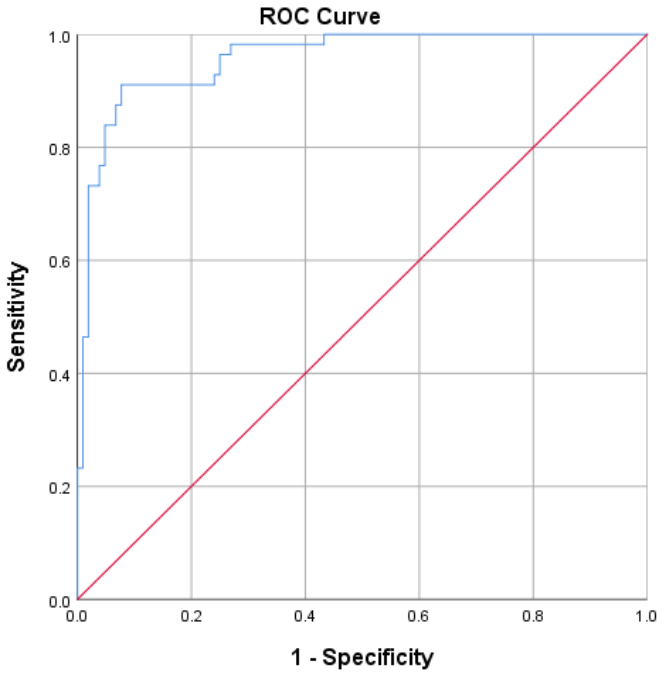


Figure 2: Relationship between pentosidine and eGFR.

There was a strong negative linear relationship between pentosidine and eGFR with a Pearson's correlation coefficient (r) = -0.778.



Area under the curve = 0.998; S.E = 0.006; p < 0.001; CI = 0.971-1.000.*

Figure 3: Receiver operating characteristics curve showing comparison between Pentosidine assay and renal status using eGFR.

Pentosidine assay had a cutoff point of 17.5690 ng/ml and sensitivity and specificity of 98.8% and 95.0% respectively.

Table 3: Cross tabulation between Pentosidine and UACR with the eGFR diagnosed groups.

Assay	DN (Positive) n = 80	Control (Negative) n = 80	Total n = 160	Stat test	P value
Pentosidine					
Positive	79 (98.8)	4 (5.0)	83 (51.9)	χ^2=140.823	***< 0.001****
Negative	1 (1.3)	76 (95.0)	77 (48.1)		

****Significant***

Using the above cutoff marks, 79 (98.8%) of the cases were diagnosed positive for DN by pentosidine assay while 1 (1.3%) was diagnosed negative. This was statistically significant (p < 0.001).

Table 4: Diagnostic accuracy of Pentosidine assay in assessing Diabetic nephropathy.

Performance variable	Pentosidine
Positive predictive value	95.18%
Negative predictive value	98.70%
Positive Likelihood ratio	19.76
Negative Likelihood ratio	0.01
Accuracy	96.86%

The positive predictive value for pentosidine assay in DN was 95.18%. The negative predictive value for pentosidine assay was 98.70%. The diagnostic accuracy of pentosidine assay in DN was 96.86%.

DISCUSSION

In this comparative cross sectional study, pentosidine, a well characterized AGE, was assessed in diabetic nephropathy patients. A total of 160 subjects participated, of which, 80 were persons diagnosed with DN using eGFR and 80 were non diabetic control persons.

Analysis of the levels of pentosidine in the cases showed a significant elevation compared to the controls. Increased plasma Pentosidine levels have been

demonstrated in patients with DN in similar literature.[24] The mean pentosidine level among the subjects was 51.87 ±25.09 ng/ml compared to 12.90 ±4.38 ng/ml for the controls. This finding however does not agree with the data from a Japanese study.[25] A study by Sell and Monnier showed significant elevation of tissue levels of pentosidine in association with end-stage renal disease in diabetic and non-diabetic subjects,[26] Furthermore, a 2011 study reported that specific AGE combinations were strongly associated with complications (specifically diabetic retinopathy).[27] Because the kidney is the main elimination site for pentosidine, serum pentosidine concentrations in patients with diabetes with overt nephropathy or those with CKD, have been recorded to be elevated.[28] A more recent study speculated that pentosidine might accelerate the development of microvascular complications both by accumulation of AGEs in the vessel walls and by causing endothelial dysfunction, as mediated by the AGE–RAGE axis activity.[29]

Pentosidine assay had a cutoff point of 17.5690 ng/ml. Pentosidine thus showed 98.8% sensitivity for the detection of DN and 95.0% specificity. This implies a high diagnostic accuracy but also high number of False Positives results would be obtained using pentosidine when compared to eGFR.

Generally, the performance of any test in a population is influenced by Positive and Negative Predictive values. Predictive values vary among populations such that PPV are higher and NPV are lower in disease prevalent areas.[30] In this study a PPV of 95.18% was recorded for pentosidine explaining the higher False Positives and the lower negative prediction of the test compared to UACR in diagnosing DN. This suggests that ~4.82% of people who test with Pentosidine in such study settings with similar characteristics as Edo State will receive False Positive results. This has remarkable implications in the diagnosis and management of diabetic nephropathy. It does not necessarily translate into rejection of the use of eGFR but adoption of pentosidine assay would lead to an improvement in the management of diabetes.

To determine whether a new test can serve as a replacement, triage instrument, or add-on test, we need more than a simple estimate of its sensitivity and specificity. The accuracy of the new testing strategy, as well as other relevant features, should be compared with that of an existing testing method. The existing testing method in this case is UACR. Although initially no correlation between Pentosidine levels and UACR were reported in literature,[31] recent publications have challenged this finding; one study reported significantly increased serum Pentosidine levels in diabetes patients with MA compared to normoalbuminuric controls[32] and another study found increased median urinary Pentosidine excretion in diabetes patients with macroalbuminuria compared to controls.[33]

The World Health Organization has defined a biomarker as any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease.[34] In summary, a clinically useful biomarker must be able to meet one of the following criteria: (i) show specificity for a certain disease (diagnostic), (ii) have prognostic value, and (iii) correlate with disease activity. This then allows treatment efficacy to be assessed. Pentosidine can correlate with disease activity and have prognostic value. Many studies suggest oxidative stress can influence the disease. However, Stress has had no effect on already established cases of DN from the study, but from literature review, it is involved in its pathogenesis. Pentosidine is also a marker of various glycation, oxidative and inflammatory disorders including diabetic retinopathy.

This may be responsible for the fact that in this study, pentosidine had a higher sensitivity and positive predictive value.

CONCLUSION

The study showed that Pentosidine level was significantly higher among patients with diabetic nephropathy. There was a significant increase in serum creatinine and urea levels in the diabetic patients compared to the non-diabetic control individuals. The study also recorded increased urinary albumin and reduced urinary creatinine among the diabetic nephropathy patients. Thus a significantly higher urinary albumin-to-creatinine ratio and lower eGFR were recorded among diabetic nephropathy patients. Serum *Pentosidine* a assay was shown to have a high diagnostic accuracy in diagnosing diabetic nephropathy.

Recommendation

Long-term, prospective study which takes into recognition suspected risks or protection factors during disease pathogenetic period will be needed to demonstrate whether elevated serum pentosidine levels are indeed predictor of nephropathy in diabetic patients. Not neglecting the current study which could serve as a foundation for clinical application and implementation of Pentosidine in DN management.

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