ISSN (Print) : 0974-6846
ISSN (Online) : 0974-5645

INDIAN JOURNAL OF SCIENCE AND TECHNOLOGY
February 2020, Vol 13(07), 832 – 839
DOI: 10.17485/ijst/2020/v13i07/149871,

Urinary Citrate: A Potential Biomarker for IgA Nephropathy

Santosh Kumar[1], C. Priscilla[1], Sreejith Parameswaran[2], Deepak Gopal Shewade[1], Rajan Sundaram[1] and Rajesh Nachiappa Ganesh [1,*]

[1]Department of Pharmacology, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, 605006, India.
[2]Department of Nephrology, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, 605006, India.

Article Type: Article

Article Citation: Santosh Kumar, Priscilla C, Sreejith Parameswaran, Deepak Gopal Shewade, Rajan Sundaram, Rajesh Nachiappa Ganesh. Urinary citrate: a potential biomarker for IgA nephropathy. *Indian Journal of Science and Technology*. 2020; 13(07),832-839.DOI:10.17485/ijst/2020/v013i07/149871

Received date: January 14, 2020

Accepted date: February 4, 2020

***Author for correspondence:** Rajesh Nachiappa Ganesh drngrajesh@gmail.com Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India

Abstract

Background: Citrate is filtered by the glomeruli and reabsorbed in tubular cells in kidney. Through this study, we have tried to explore citrate as a diagnostic tool for IgA nephropathy. **Methods**: We recruited 35 IgA nephropathy (IgAN) patients and 15 healthy controls (HC). 30 ml urine sample collected from each study participant. Solid phase extraction method used for urine purification. Liquid chromatography attached with mass spectrometry (LC–MS/MS) used for the citrate concentration determination. Logistic regression method used for diagnostic model prediction. **Findings:** Urinary citrate level was higher in IgAN patients by more than two and half times in comparison to HC. We made logistic regression model with citrate, urine protein, estimated glomerular filtration rate (eGFR), urine pH, systolic and diastolic blood pressure as variables. Citrate with urine protein was found to be the best fit statistical model with area under curve 0.77 and sensitivity and specificity more than 0.70 and 0.80, respectively. **Applications:** Urinary citrate with urine protein can be used for the early prediction of IgAN.

Keywords: IgAN, Citrate, Urine Protein, Kidney Stone.

1. Introduction

Urinary citrate is seen as a kidney stone prohibition agent [1]. Citrate forms soluble complexes with calcium, thus prevents the formation of calcium stone in kidney [2]. It is filtered at the glomerulus and reabsorbed in the proximal tubules [3]. Excretion of citrate in urine depends on the rate of citrate absorption from the glomerulus filtration and on the metabolism occurs in the proximal tubule in kidney [4].

In this study, we have quantified the urinary citrate in IgA nephropathy (IgAN) patients and healthy controls by liquid chromatography attached mass spectrometry (LC/LC–MS) method. IgAN is a glomerular disease whose pathogenesis is still partially known [5]. It is a common glomerular disease worldwide and prone to the young adults [6–7]. Its progression is slow and reaches to the end stage renal diseases in 10–20 years [8].

IgAN is caused by the deposition of IgA molecules in glomeruli and can diagnose by the fluorescence-based microscopy examination of kidney biopsy [9–10]. Kidney biopsy is a surgical procedure and not advised at the initial stage in kidney patients, which leads to late diagnosis of IgAN [11]. IgA deposition in glomeruli destroys the filtration process in nephron and it may affect the citrate excretion in urine.

Here, we have tried to find the citrate concentration in urine for IgAN and healthy controls to see the differences and further to see the potential of citrate as a diagnostic tool.

2. Materials and Methods

This study was carried at a tertiary health care center. We took institute human ethical clearance before recruiting participants as diseased and healthy. The patients who diagnosed with IgAN after renal biopsy confirmation were recruited for the study. The IgAN patients with HIV, Cancer, and other autoimmune diseases were not considered for the study and excluded. The healthy participants were recruited on age and sex matched criteria. Only those healthy participants were selected in the study whose serum creatinine was lesser or equal to 1 mg/dl, systolic and diastolic blood pressure were less or equal to 120 and 80 mmHG, respectively, the urine protein level was 0 by dipstick method. The age group of the participants was 15–70 years. 35 IgAN patients and 15 healthy control were recruited as study participants following inclusion and exclusion criteria.

2.1. Sample Collection

Written informed consent was taken from each participant before sample collection. 30 ml urine was collected from each diseased group participant on their arrival at the nephrology department in the institute. The diseased group participants were not given any prior instruction on diet control for the purpose of sample collection. The sample was collected at random fulfilling inclusion and exclusion criteria. Healthy participants too were not given any prior dietary restriction. 30 ml urine collected from age and sex matched healthy participants following inclusion and exclusion criteria. The urine was collected into sodium azide coated container and carried immediately to the processing laboratory. Urine pH was measured with pH electrode (Mettler Toledo, LE410). The urine was centrifuged at 3000 *g* value for 10 min at 4 degree centigrade and supernatants were stored in aliquots at −80 degree centigrade immediately for further processing.

2.2. Solid Phase Extraction

The stored samples were centrifuged at 12,000 *g* value for 10 min at 4 degree centigrade. Urine supernatants were further processed for purification. Solid phase extraction (SPE)

method was used for purification of the sample. OASIS@HLB 1cc (30 mg) extraction cartridge (Waters 094225) was used for solid phase extraction. HPLC grade water and mass spectrometry grade methanol used throughout the experiment. Solid phase extraction was performed in five stages. 1 ml methanol (100%) followed by 1 ml water than 1 ml urine sample was inserted into cartridge column. It was washed with 1 ml 5% methanol and finally 1 ml methanol (100%) used and the extract was used for the LC/MS–MS input sample.

2.3. Metabolite Quantification

Standard curve was made from reference standard material of citrate (96068, Sigma Aldrich). Citrate concentration was prepared (in methanol) as 1 µg/µl, 5 µg/µl, 10 µg/µl, 15 µg/µl, and 20 µg/µl to make standard curve. Liquid chromatography (LC) process was performed by Waters Acquity (UPLC) – 2695 and mass spectrometry (MS) was performed by Waters -2998 instrument. 0.1% formic acid with water used as solvent. C18 column was used at 30 degree centigrade. 10 µl sample was used as input sample for LC. Column flow was 0.3 ml/minute. MS flow rate was 5 µl/minute. Cone voltage maintained at 30 V. Duration was set at 5 min. Electron spray negative (ES–) ion mode method was performed. Mass lynx 4.1 software was used for analysis and quantifying metabolites. Fold change analysis was performed by log10 (IgANm/HCm) method, where IgANm and HCm represent metabolite quantity in IgAN and HC participants, respectively.

2.4. Histopathological Analysis

Native kidney biopsies of IgAN patients were analyzed and reported by immunofluorescence-based light microscopy. MEST-C (M- Mesangial hypercellularity, E- Endocapillary hypercellularity, S- Segmental glomerulosclerosis, T- Tubular atrophy/Interstitial fibrosis, C- Crescents) score according to Oxford classification of IgA nephropathy was used and biopsy findings were documented[12].

2.5. Statistical Analysis

Shapiro Wilk normality test was performed for the data distribution. *t*-test and Wilcoxon's rank sum test were used for study group differentiation. Spearman rank correlation was performed for correlation analysis. Logistic regression method used to build statistical model to validate the diagnostic importance of citrate. Akaike information criteria (AIC) and Bayes information criteria (BIC) used to select the best fit regression model. Receiver operating characteristic (ROC) analysis performed using pROC package [13]. p Value less than 0.05 was considered as significant. All the statistical tests performed using R version 3.6.2 [14].

2.6. Ethical approval

"All procedures performed in this study involving human participants were in accordance with the ethical standards of the institute research and human ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

The study was started after the approval of institute human ethics committee (JIP/IEC/SC/2015/19/785). Informed written consent was taken from all individual participants included in the study.

3. Results

The IgAN patients found to be young and of almost equal sex ratio (Table 1). The blood pressure of the IgAN patients was in hypertension stage -1 (Table 1). The mean (with standard deviation) urine pH value of IgAN and HC groups were 6.31 (±0.38) and 6.64 (±0.27), respectively. There was a statistically significant difference in urine pH between the groups with p value 0.002. The average citrate concentration in IgAN patients was more than 2.5 times than the healthy control. The concentration of citrate was statistically significant different in both groups with p value 0.002 (Figure 1). The fold change for citrate in IgAN patients in comparison to HC was 0.45. We made logistic regression based diagnostic model to assess citrate as diagnostic marker. Out of 41 logistic regression models made from the six predictive variables i.e. systolic blood pressure, diastolic blood pressure, eGFR, urine pH, urine protein (dipstick method), and citrate, we selected 10 models based on citrate as one of the variables. Out of citrate containing models, we selected three models based on least AIC, BIC, and sum of AIC and BIC scores. AIC, BIC, and AIC + BIC scores for the model-1 (variable = urine protein and citrate), model-2 (variables = eGFR, urine protein, and citrate), and model-3 (systolic blood pressure, diastolic blood pressure, eGFR, urine pH, urine protein, and citrate) were 26.36, 32.10, and 58.46; 25.76, 33.40, and 59.16; and 23.36, 34.83, and 58.19, respectively. The ROC test performed for the selected statistical models and area under ROC curve (AUC), sensitivity (SE), and specificity (SP) were calculated. The AUC, SE, and SP for model-1, model-2, and model-3 were 0.777, 0.714, and 0.800; 0.438, 0.510, and 0.530; and 0.627, 0.570, and 0.730, respectively. The best fit statistical model was selected based on least AIC + BIC score and highest AUC + SE + SP value (Figure 2). We performed Spearman's rank correlation test on all the variables taken together including histopathological parameters. We could not find any significant association ($\rho > 0.35$ and $p < 0.05$) of citrate with systolic blood pressure, diastolic blood pressure, urine protein, urine pH, eGFR, M, E, S, T, and C.

TABLE 1. Characteristics of study participants

	IgAN	HC
Age (years)	29.23 (9.38)	28.46 (7.45)
Male	54%	53%
Female	46%	47%
eGFR (ml/min per 1.73 m^2)	36.00 (14.50–82.00)	128 (116.50–145.50)
Citrate (µg/µl)	197.7 (111.80–376.10)	84.30 (48.95–125.00)
Systolic blood pressure (mmHg)	125 (17.84)	112.2 (5.27)
Diastolic blood pressure (mmHg)	82.06 (11.57)	75.87 (2.5)
Urine pH	6.31 (0.38)	6.64 (0.27)

eGFR: estimated glomerular filtration rate; HC: healthy controls; IgAN: IgA nephropathy.

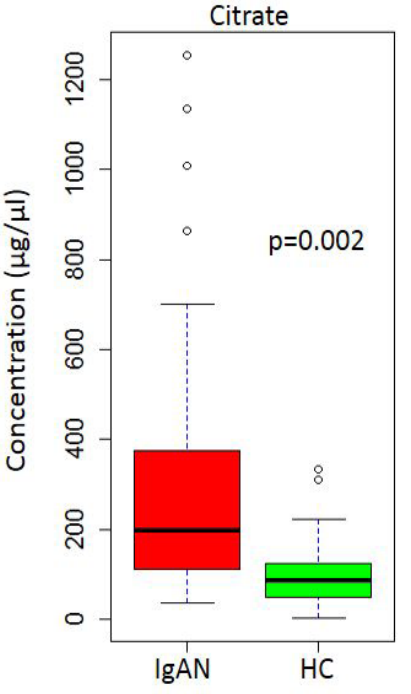

FIGURE 1. Citrate concentration in urine. HC: healthy control; IgAN: IgA nephropathy.

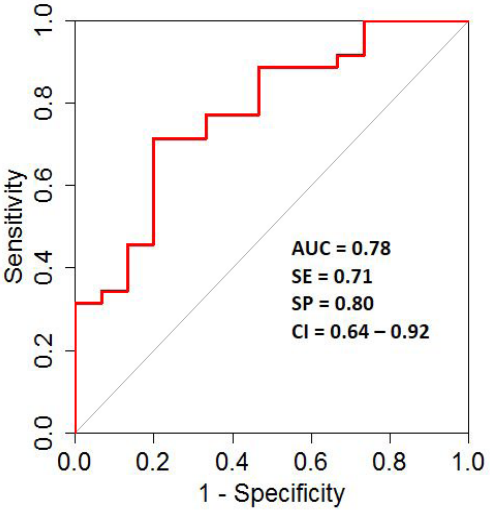

FIGURE 2. Diagnostic validity of urinary citrate and protein for IgAN. AUC: area under receiver operating characteristic curve; CI: confidence interval; IgAN: IgA nephropathy; SE: sensitivity; SP: specificity (DeLong's method).

4. Discussion

Low citrate excretion in urine increases the risk of calcium stone formation in kidney [2]. More than 50% of the filtrate citrate from glomeruli is reabsorbed in the proximal tubules [15]. If we see citrate filtration by the glomeruli and reabsorption in proximal tubular in the context of IgAN, we find that there are two possible conditions involved to alter the citrate excretion in urine. A disturbed glomerular filtration is the common feature of IgAN and hence common to both the conditions. Blood and protein in urine are the result of disturbed filtration at glomeruli and we assume that citrate is also excreted more in quantity in urine, which is supported by our result of increased citrate concentration in urine. In the first condition, citrate absorption in proximal tubular cells is normal in the absence of tubular cell injury. In the second condition, there is injury at the proximal tubular cells which results in disturbed absorption of citrate resulting into more excretion of citrate in urine than the first condition. Proximal tubular cell injury also causes IgAN to turn into end stage renal disease (ESRD) rapidly [16]. In this case, the higher citrate level in urine makes IgAN more progressive towards ESRD. There was a study reported from Italy by Del Coco et al., where they found citrate to be in lower concentration in urine than the healthy controls in nuclear magnetic resonance (NMR) based experiment [17]. In a study conducted at Johns Hopkins Hospital and Cincinnati Children's Hospital and Medical Center, the authors reported lower citrate concentration in stage five of Lupus Nephritis (LN) than the stage three LN patients [18]. In a study reported from Iran, citrate concentration in Membranous glomerulonephritis was found lower in comparison to healthy controls in an NMR-based study [19]. Our findings are opposite to the previous studies. In a case control study where specified groups are taken from the same population of different subset, metabolite concentration may vary in each group for different population, but not comparatively in groups (subsets) from the same population and thus we rule out the population difference effect for our opposite directional result. There was no IgAN patient found in our study group having case history of kidney stone. It further validates our findings of high urinary citrate in IgAN patients. The healthy control group in our study was restricted with serum creatinine, systolic and diastolic blood pressure levels. In this condition, these parameters are biased and we did not include these controlled variables independently for disease progression or to see the differences between the groups. Citrate and urine pH were independent parameters where no dietary instruction was given to the study groups. Our result showed that model-1 i.e. citrate and urine protein model was the best fit model for the diagnosis of IgAN. On the pattern of hematuria and urinary protein, citrate too can be added as routine test for disease diagnosis. A regular urinary citrate test can serve two purposes. The lower urine citrate concentration can indicate the risk of renal stone formation where higher concentration could be a sign of IgAN development. A multi-centric study with higher sample size can be done to decide the cut off value of citrate for the risk of kidney stone (less than the lower cutoff value) and IgAN (more than the upper cut off value) prediction. Furthermore, a longitudinal study can be formed to see the association of urine citrate level with the disease progression.

5. Conclusion

Urine citrate has potential to be used as a non-invasive biomarker for IgAN. Urine citrate with urine protein can early predict the IgAN. It will reduce the dependency of kidney biopsy to diagnose the IgAN.

Conflict of Interest

None.

Acknowledgement

This work was supported by Science and Engineering Research Board (SERB), Government of India (File number – EMR/2016/003382) and Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India.

References

1. Unwina RJ, Capasso G, Shirley DG. An overview of divalent cation and citrate handling by the kidney. *Nephron Physiology*. 2004, 98(2), 15–20. https://doi.org/10.1159/000080259
2. Menon M, Mahle CJ. Urinary citrate excretion in patients with renal calculi. *The Journal of Urology*. 1983, 129(6), 1158–1160. https://doi.org/10.1016/S0022-5347(17)52618-X
3. Caudarella R, Vescini F, Buffa A, Stefoni S. Citrate and mineral metabolism: kidney stones and bone disease. *Front Bioscience*. 2003, 8, s1084-s1106. http://doi.org/10.2741/1119
4. Zacchia M, Preisig P. Low urinary citrate: an overview. *Journal of Nephrology*. 2010, 16, S49–S56. External source: https://www.ncbi.nlm.nih.gov/pubmed/21170889
5. Rodrigues JC, Haas M, Reich HN. IgA nephropathy. *Clinical Journal of the American Society of Nephrology*. 2017, 12(4), 677–686. https://doi.org/10.2215/CJN.07420716
6. Yu HH, Chiang BL. Diagnosis and classification of IgA nephropathy. *Autoimmunity Reviews*. 2014, 13(4), 556–559. https://doi.org/10.1016/j.autrev.2014.01.030
7. Schena FP, Nistor I. Epidemiology of IgA nephropathy: a global perspective. *Seminars in Nephrology*. 2018, 38(5), 435–442. https://doi.org/10.1016/j.semnephrol.2018.05.013
8. Wyatt RJ, Julian BA. IgA nephropathy. *The New England Journal of Medicine*. 2013, 368(25), 2402–2414. https://doi.org/10.1056/NEJMra1206793
9. Tesar V, Hruskova Z. Understanding Histolopathologic characteristics to predict renal outcomes in lupus nephritis. *Clinical Journal of the American Society of Nephrology*. 2017, 12(5), 711–712. https://doi.org/10.2215/cjn.03490317
10. Bagchi S, Lingaiah R, Mani K, Barwad A, Singh G, Balooni V. Significance of serum galactose deficient IgA1 as a potential biomarker for IgA nephropathy: a case control study. *PLoS One*. 2019, 1–14. https://doi.org/10.1371/journal.pone.0214256
11. Barratt J. IgA Nephropathy. *Journal of the American Society of Nephrology*. 2005, 16(7), 2088–2097. https://doi.org/10.1681/ASN.2005020134
12. Trimarchi H, Barratt J, Cattran DC, Cook HT, Coppo R, Haas Ml. Oxford classification of IgA nephropathy 2016: an update from the IgA nephropathy classification working group. *Kidney International*. 2017, 91(5), 1014–1021. https://doi.org/10.1016/j.kint.2017.02.003

13. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics*. 2011, 12, 77. https://doi.org/10.1186/1471-2105-12-77
14. A language and environment for statistical computing. R Foundation for Statistical Computing: Vienna, Austria. https://www.R-project.org/. Date accessed: 2019.
15. Hamm LL. Renal handling of citrate. *Kidney International*. 1990, 38(4), 728–735. https://doi.org/10.1038/ki.1990.265
16. Leung JCK, Lai KN, Tang SCW. Role of mesangial-podocytic-tubular cross-talk in IgA nephropathy, *Seminars in Nephrology*. 2018, 38, 485–495. https://doi.org/10.1016/j.semnephrol.2018.05.018
17. Del LC, Michael A, Mariapina D, Fabio S, Francesco P, Francesco PF et al. A proton nuclear magnetic resonance-based metabolomic approach in IgA nephropathy urinary profiles. *Metabolomics*. 2013, 9(3), 740–751. https://doi.org/10.1007/s11306-012-0489-2
18. Romick-Rosendale LE, Brunner HI, Bennett MR, Mina R, Nelson S. Identification of urinary metabolites that distinguish membranous lupus nephritis from proliferative lupus nephritis and focal segmental glomerulosclerosis. *Arthritis Research & Therapy*. 2011, 13(6), 199. https://doi.org/10.1186/ar3530
19. Taherkhani A, Kalantari S, Oskouie AA, Nafar M, Taghizadeh M, Tabar K. Network analysis of membranous glomerulonephritis based on metabolomics data. *Molecular Medicine Reports*. 2018, 18(5), 4197–4212. https://doi.org/10.3892/mmr.2018.9477