

Original Research Article

Role of urinary nitrites in predicting steroid responsiveness of nephrotic syndrome: a study conducted in tertiary care center

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ABSTRACT

Background: Nephrotic Syndrome (NS) is a common chronic disorder, characterized by alterations of selective permeability at the glomerular capillary wall, resulting in its inability to restrict the urinary loss of protein. Urinary nitrite excretion serves as a useful investigation in differentiating between steroid responsive and steroid resistant nephrotic syndrome. The aim of the study was to assess the relation between urinary nitrite levels and steroid responsiveness in nephrotic syndrome in children.

Methods: 76 children were enrolled in the study suffering with nephrotic syndrome of which 58 children were Steroid Sensitive (SSNS) and 18 were Steroid Resistant (SRNS). 25 children were enrolled as controls. The urinary nitrites were estimated in these subjects and the results were analyzed.

Results: All the control subjects were tested negative for urinary nitrites. After achieving remission with steroids, out of 58 SSNS subjects' 27 subjects tested positive for urinary nitrites, remaining 31 tested negatives for the same. Of the 18 SRNS subjects 1 subject tested positive for urinary nitrites remaining 17 subjects were tested negative for the same.

Conclusions: The findings of present study suggest that urinary nitrite excretion is increased in patients with steroid responsive nephrotic syndrome. The urinary nitrite estimation has low NPV and high PPV in predicting steroid responsiveness.

Keywords: Nephrotic syndrome, Steroid resistant, Steroid responsive, Urinary nitrites

INTRODUCTION

Nephrotic range proteinuria is defined as proteinuria exceeding 40 mg/m² per hour or spot (random) urinary protein-to creatinine ratio exceeding 2 mg/mg of creatinine. The triad of clinical findings associated with nephrotic syndrome arising from the large urinary losses of protein are hypoalbuminemia (<2.5 g/dl), edema, and hyper lipidemic. Estimates on the annual incidence of nephrotic syndrome range from 2-7 per 100,000 according to global statistics.¹ There is epidemiological

evidence of a higher incidence of nephrotic syndrome in children from south Asia.² More than 80% patients with nephrotic syndrome show Minimal Change Disease (MCD) characterized by normal renal histology on light microscopy. The remaining is contributed by Focal Segmental Glomerulosclerosis (FSGS) and Mesangioproliferative Glomerulonephritis (MesPGN). MCD and FSGS are often considered to represent the same pathophysiological process. Children with onset of uncomplicated nephrotic syndrome between 1 and 8 yr of age are likely to have steroid responsive MCNS, and

steroid therapy may be initiated without a diagnostic renal biopsy. Children who fail to respond within 8 weeks of steroid therapy are considered steroid resistant. Children with steroid-resistant nephrotic syndrome; generally, have a much poorer prognosis. These children develop progressive renal insufficiency, ultimately leading to end-stage renal disease requiring dialysis or kidney transplantation.

In clinical practice, the best prognostic indicator for NS is whether or not the disease responds to steroid treatment.³ Patients with SSNS or SRNS have a similar clinical manifestation, and there is no specific laboratory indicator to distinguish these two clinical entities.⁴ Pathological evaluation of renal cortical tissue, by means of a renal biopsy, has traditionally been used to detect a distinction between SSNS and SRNS.⁴ The pathological correlations to SSNS and SRNS are minimal change disease and focal segmental glomerulosclerosis, respectively. However, these histological diagnoses aren't always parallel to patients' clinical response to treatment. Identification of noninvasive biomarkers that accurately distinguish SSNS from SRNS would be most beneficial to the patients with SRNS, preventing their exposure to high dose, yet ineffective steroid courses. Because inappropriate use of steroids in children is especially hazardous, it would be important to detect this small proportion of SRNS by simple tests. Some authors described the role of urinary nitrite as method to predict steroid response in idiopathic nephrotic syndrome.

The pathogenesis of nephrotic syndrome is unclear. Shalhoub et al, postulated the role of lymphokines in increasing the permeability of the glomerular basement membranes.⁵ Clinical observations suggest that lipoid nephrosis is produced by a systemic abnormality of T cell function resulting in the secretion of a circulating chemical mediator toxic to an immunologically innocent glomerular basement membrane.⁵ T cell hybridomas derived from the T cells of a patient with Minimal Change Nephrotic Syndrome (MCNS) made a glomerular permeability factor. Later on, vascular permeability factor, and interleukin 2 were implicated in its pathogenesis.^{5,6} Although organic nitrates were known to be potent vasodilators for a century, the role of nitric oxide in the pathogenesis of nephrotic syndrome has been postulated in 1996 by Trachtman et al.⁷

Nitric Oxide (NO) is synthesized in the body from L-arginine by enzyme nitric oxide synthase. It is a highly reactive free radical gas that easily decomposes into nitrite and nitrate in biological fluids. The concentration of these can be measured and used as markers of nitric oxide production.⁷ Studies have been published related to increased glomerular synthesis of nitric oxide in several well characterized models of immune glomerulonephritis in rats.⁸⁻¹⁰ They tested the hypothesis that nitric oxide synthesis by the kidney is increased in children with primary nephrotic syndrome. They examined the urinary excretion of nitrite, a stable metabolite of nitric oxide,

using the Griess reaction, in children with nephrotic syndrome. They found that in comparison with healthy children, patients with minimal change nephrotic syndrome had increased urinary nitrite excretion regardless of whether the disease was in relapse or remission ($p < 0.025$). In contrast, urinary nitrite excretion was similar in control subjects and patients with focal segmental glomerulosclerosis or IgA nephropathy. These findings indicate that measurement of urinary nitrite excretion may be a useful test to help discriminate between minimal change nephrotic syndrome and focal segmental glomerulosclerosis.⁷

Similar kind of observations made in the study by N K Dubey et al, in year 2000.¹¹ They observed urinary nitrite excretion is increased in children with nephrotic syndrome, particularly in steroid responsive nephrotic syndrome. This increase in urinary nitrite excretion is irrespective of the state of the disease. Steroid therapy has no effect on urinary nitrite excretion. Urinary nitrite excretion serves as a useful investigation in differentiating between steroid responsive and steroid resistant nephrotic syndrome. That nitric oxide production is not an important and sole cause of the disease is further substantiated by near normal level of urinary nitrite found in children with steroid resistant nephrotic syndrome in their study.¹¹ Similar observations were made in studies done by Serdaroglu E, Furuşu et al, also found a decreased expression of endothelial nitric oxide synthase (eNOS) in patients with IgA nephropathy and lupus nephritis suggesting diminished physiological effect of eNOS (decreased generation of nitric oxide) in damaged glomeruli.^{3,12} This could be another explanation for near normal value found in case of IgA nephropathy and focal segmental glomerulosclerosis in their study.

Although renal biopsy is the definitive investigation in kidney disorders and is particularly helpful in distinguishing Steroid-Responsive Nephrotic Syndrome (SRNS) from focal glomerulosclerosis (FGS), it is attended by a small risk to the patient. Accordingly, noninvasive tests have been used to predict the response to steroids and the underlying renal histologic diagnosis in nephrotic syndrome.¹³

As it is simple and inexpensive to do in hospital setting, authors studied the role of urinary nitrites in predicting steroid responsiveness of idiopathic nephrotic syndrome.

Research question is the measurement of urinary nitrite levels in children with nephrotic syndrome helpful in predicting the steroid responsiveness?

Hypothesis is urinary nitrite excretion is increased in children with steroid responsive nephrotic syndrome.

Objective was to estimate urinary nitrite levels in children with nephrotic syndrome and correlate them with clinical response to steroid therapy.

Aim was to assess the relation between urinary nitrite levels and steroid responsiveness in nephrotic syndrome in children.

METHODS

Study design was prospective observational study. Niloufer Institute for Child Health, Hyderabad, India. Study period was October 2014 To October 2015 for collection of samples and November 2015 for final analysis and compilation. Children of age group 2- 8 years with nephrotic syndrome.

Nephrotic syndrome

A clinical syndrome of massive proteinuria defined by

- Urine protein excretion greater than 40 mg/m²/hour on a timed urine collection or an early morning urine protein creatinine index of >200 mg/mmol;
- Hypoalbuminaemia of <25 g/l,
- Oedema.
- Hypercholesterolaemia is not needed in definition.

Remission

Urine albumin nil or trace for 3 consecutive early morning specimens.

Relapse

Urine albumin 3+ or 4+ (or proteinuria >40 mg/m²/h) for 3 consecutive early morning specimens, having been in remission previously.

Frequent relapses

Two or more relapses in initial six months or more than three relapses in any twelve months.

Steroid dependence

Two consecutive relapses when on alternate day steroids or within 14 days of its discontinuation.

Steroid resistance

Absence of remission despite therapy with daily prednisolone at a dose of 2 mg/kg per day for 8 weeks.

Exclusion criteria

- Age <2 year, and >8years
- Gross haematuria
- Systemic diseases such as lupus nephritis, Henoch Scholen purpura and poly arteritis nodosa
- Child with urinary tract infection (culture positive)

Controls

Children of same age group attending the hospital for other reasons. Controls can be defined as: a) No care giver reported history of urinary symptoms like oliguria, dysuria and haematuria b) not a known case of renal disease.

Exclusion criteria for controls

- Child with urinary tract infection.

Sample size was for cases - 58 SSNS cases and 18 SRNS cases and for controls- 25.

Ethics committee approval was taken from the institutional ethics committee of Osmania Medical College. Informed consent was taken from the parent/guardian. The Xerox copy of the information and consent form were provided to the parent/guardian. In case, if the parent/guardian is illiterate, authors also took the signature of a witness who may be a legally acceptable representative or an impartial witness. Children with nephrotic syndrome are enrolled for study. All are investigated & treated according to the ISKDC guidelines. The base line characteristics like age, sex, registration number, urinary protein value, serum albumin, serum cholesterol was noted. Urinary nitrite levels were estimated once the diagnosis is confirmed and after the remission in steroid sensitive subjects. Urinary nitrite levels estimated once in steroid resistant cases irrespective of state of the disease. Urinary nitrite levels were estimated in control subjects. Using all these data a master chart is prepared. Statistical analysis was done using the software SPSS version 21 software with help of a qualified statistician. p value of <0.05 was taken as significant.

Method of urinary nitrite estimation

Mid stream clean catch sample, in a sterile container is collected, and analysed within 2 hours of collection. Sample is analysed using CLINITEK Status+ Analyzer, with software version 2.40 which gives the results as positive and negative.

Principle

In pH of <7, nitrite in the urine reacts with p-arsanilic acid to form a diazonium compound. This diazonium compound couples with 1, 2, 3, 4-tetrahydrobenzo (h) quinolin-3-ol to produce a pink color.

Reagent

A 1.4% w/w p-arsanilic acid; 1.3% w/w 1,2,3,4-tetrahydrobenzo(h) quinolin-3-ol; 10.8% w/w buffer; 86.5% w/w nonreactive ingredients.

Sensitivity was nitrite 0.06-0.1 mg/dl.

False negative nitrites may occur with 1) ascorbic acid levels > 25 mg/dL at low nitrite levels; 2) lack of dietary nitrate; 3) high urine specific gravity. None of the subjects were on vitamin C supplements. None of them are severely malnourished and all are on high protein diet, so dietary nitrate levels presumed to be adequate.

Methods of analysis

The continuous variables like age, serum albumin, serum cholesterol was compared using unpaired t test. A standard 2x2 table constructed and analysed for sensitivity and specificity of urinary nitrites for steroid responsiveness. A Receiver Operating Characteristics (ROC), chi square test also done. Wilcoxon signed ranks test done between the categorical variables.

RESULTS

Age and sex distribution of subjects

During the study period, total of 76 children with idiopathic nephrotic syndrome were enrolled for the study, of these 58 children were steroid sensitive, remaining 18 were steroid resistant (Figure 1). The controls in the study were 25 children.

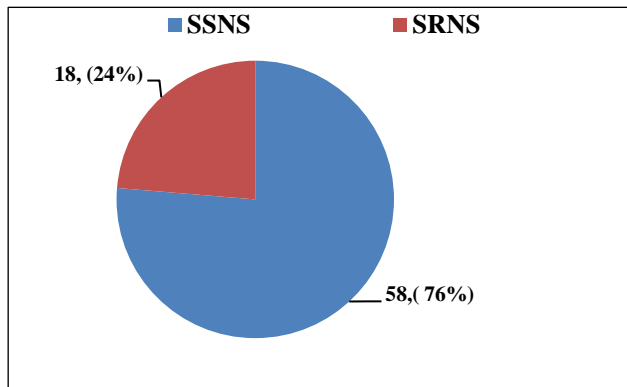


Figure 1: Distribution of cases based on steroid sensitivity.

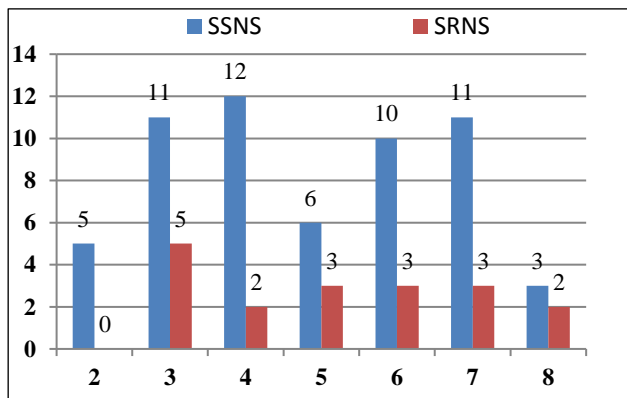


Figure 2: Age wise distribution of cases.

All the subjects were ranged from 2 to 8 years of age with median age of 5 years. SSNS subjects ranged from 2 to 8 years with median age of 4 years, and SRNS subjects ranged from 3 to 8 years with median age of 5 years (Figure 2).

Among all the 76 subjects, 34 were female and 42 were male with male to female ratio of 1.23:1

Table 1: Sex distribution of groups.

Sex	SSNS	SRNS
Male	34	8
Female	24	10

Out of 58 steroid sensitive subjects, 34 were male (58.6%), remaining 24 were females (41.4%). Out of 18 case of SRNS, 8 were male (44.4%), remaining 10 were female (55.6%) (Table 1).

Biochemical characteristics of subjects

The mean serum albumin levels of SSNS subjects are 2.07 g/dl, whereas the mean value of SRNS subjects is 1.92 g/dl. There is no statistically significant difference between the two groups' serum albumin levels. The mean serum cholesterol levels of SSNS subjects are 294.6 mg/dl, whereas the mean value in SRNS subjects is 303.6 mg/dl. There is no statistically significant difference between the two groups serum cholesterol levels (Table 2).

Table 2: Comparison of biochemical characteristics.

	SSNS	SRNS	Statistical significance?
Serum albumin	2.07 g/dl	1.92 g/dl	No (p=0.1)
Serum cholesterol	294.6 mg/dl	303.6 mg/dl	No (p= 0.4)

Characteristics of controls

A total of 25 controls were taken into the study. Of these control subjects 12 were female subjects, 13 were male subjects. Controls were ranged from 3 to 8 years of age, with median age of 5 years and are comparable with the study group.

Results of urinary nitrites estimation

All the control subjects were tested negative for urinary nitrites. Of the 58 SSNS subjects, 34 subjects tested positive for urinary nitrite and remaining 24 were tested negative for the same at the admission (i.e. before achieving remission/during relapse). After achieving remission with steroids, out of 58 SSNS subjects, 27 subjects tested positive for urinary nitrites, remaining 31 tested negatives for the same. Of the 18 SRNS subjects 1

subject tested positive for urinary nitrites remaining 17 subjects were tested negative for the same (Table 3).

Table 3: Results of urinary nitrites.

	Urinary nitrites - positive	Urinary nitrites - negative	Total
SSNS (before remission)	34	24	58
SSNS (after remission)	27	31	58
SRNS	1	17	18

Correlation of urinary nitrites with steroid responsiveness

Using the above results, a 2x2 table is constructed (Table 4), with urinary nitrite value as the test and whether the child responded to steroid therapy or not as the final outcome.

Table 4: 2X2 table.

Urinary nitrites	Steroid sensitive		Total
	Yes	No	
Positive	34	1	35
Negative	24	17	41

Using this table, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of urinary nitrites in predicting steroid responsiveness were calculated (Table 5).

Table 5: Role urinary nitrites in predicting steroid responsiveness.

Sensitivity	58.6%
Specificity	94.4%
PPV	97.1 %

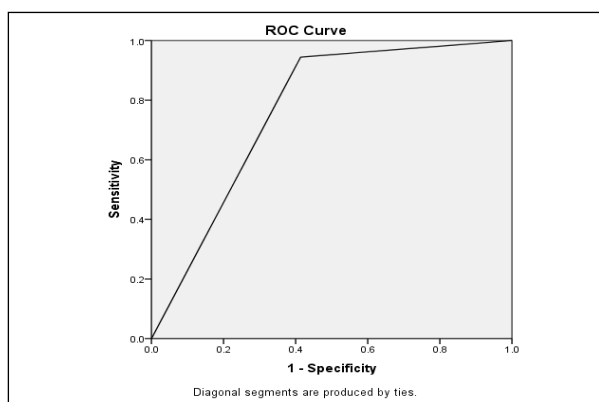


Figure 3: ROC curve.

The chi square test (fisher’s exact test) did between the two groups show the p value as 0.001, which is

statistically significant association between urinary nitrites and steroid responsiveness. ROC curve is plotted using the 2x2 table (Figure 3). The area under curve (AOC) is 0.729. Which implies there is fair agreement between the test used and the output.

The urinary nitrite values in SSNS subjects before and after remission were correlated using the Wilcoxon Signed Ranks Test which showed the p value as 0.67 and is statistically not significant.

DISCUSSION

The basic characteristics of study subjects including age and sex distribution are comparable between the two groups. The biochemical characteristics including serum albumin, serum cholesterol is comparable between the two study groups. The unpaired t test done showed the p value more than 0.05. There is no statistically significant difference between two study groups for these base line characteristics. Urinary nitrites estimation has 58.6% sensitivity (true positive rate) for detecting the steroid responsiveness. This means only 58.6% cases which are steroid sensitive, tested positive for urinary nitrites. In remaining 41.4% cases though they are steroid sensitive urinary nitrites were negative.

Thus, urinary nitrites have poor sensitivity for predicting steroid responsiveness. The possible explanations include

- Pathogenesis of glomerular permeability in cases of SSNS may not be entirely mediated through nitric oxide.
- The excreted nitrite levels may not be detectable with the test used in the study (0.06-1 mg/dl).

The specificity of urinary nitrite in detecting steroid responsiveness is 94.4%. The 17 cases out of 18 SRNS tested negative for urinary nitrites, this is comparable with the control subjects. All 25 controls tested negative for urinary nitrites. This implies that there is no or not detectable nitrite levels in normal children or in SRNS cases. The possible mechanism of glomerular injury in case of SRNS is different from that of SSNS and it is possibly not nitric oxide mediated. The PPV value of urinary nitrites in detecting steroid responsiveness is 97.1%. This implies when the subject tests positive for urinary nitrites there is high probability that it is going to be steroid sensitive. The low NPV implies that negative urinary nitrite value is not much significant in predicting steroid resistance. In present study the urinary nitrites showed high specificity and high positive predictive value, and low sensitivity and low negative predictive value in predicting steroid responsiveness. A positive urinary nitrite value highly predicts that child will go into the remission with steroid therapy, whereas negative value of the same cannot predict against it with high probability. The low sensitivity of urinary nitrite also means that, though child has negative urinary nitrites, a therapeutic trial of corticosteroid is still recommended.

The similar kind of study done by N K Dubey et al, showed that increased urinary nitrite estimation in steroid sensitive subjects.¹¹ Summary of their study are as in following Table 6.

Table 6: Comparison of urinary nitrite in N K dubey et al, study.¹¹

	Urinary nitrite value(mmol/mg)	Urinary nitrite value of controls	P value
Steroid sensitive	2427±1548	1060±388	<0.005
Steroid resistant	1125±316.5	1060±388	0.6

In present study the urinary nitrite levels were reported as positive or negative with sensitivity of the urine analyzer being 0.06-0.1 mg/dl, whereas in NK Dubey et al, study 11 the absolute values of urinary nitrites were taken. The statistical analysis was done by using the mean values of urinary nitrites in their study. They observed that there is significant association between urinary nitrite levels and steroid responsiveness but not able to conclude the usefulness of this test in terms of sensitivity and specificity etc. In this study, by measuring the urinary nitrites as positive and negative, we were able to estimate the usefulness of urinary nitrites in terms of sensitivity, specificity, PPV, and NPV. Trachtman et al, did the similar kind of study and found that steroid sensitive nephrotic syndrome subjects have high levels of urinary nitrite excretion compared to steroid resistant cases.⁷ The sensitivity and specificity in their study were 45% and 92% respectively (Table 7).

Table 7: Comparison with trachtman et al, study.⁷

	Trachtman et al ⁷	Present study
Sensitivity	45%	58.6%
Specificity	92%	94.4%

These observations were similar to present study. That nitric oxide production is not an important and sole cause of the disease is further substantiated by near normal level of urinary nitrite found in children with steroid resistant nephrotic syndrome in the present study. Similar observations were made in earlier studies.¹² Furuu et al, also found a decreased expression of endothelial Nitric Oxide Synthase (eNOS) in patients with IgA nephropathy and lupus nephritis suggesting diminished physiological effect of eNOS (decreased generation of nitric oxide) in damaged glomeruli. This could be another explanation for near normal value found in cases of steroid resistant nephrotic syndrome in the present study. The correlation of urinary nitrite values in SSNS subjects before and after remission done, out of 58 SSNS subjects 34 were positive for urinary nitrites before remission and 27 were positive for the same after remission. All 24 subjects who are negative for urinary nitrites remained same after remission. 7 subjects who are positive for urinary nitrites

before remission became negative for the same after achieving remission (Table 8).

Table 8: Comparison of SSNS before and after remission.

	Urinary nitrites - positive	Urinary nitrites - negative	Total
SSNS (before remission)	34	24	58
SSNS (after remission)	27	31	58

The two groups were compared using Wilcoxon Signed Ranks Test which showed the p value as 0.67 and is statistically not significant. In NK Dubey et al, study and Trachtman et al, study also urinary nitrite excretion does not show any statistically significant variation before and after the remission in SSNS subjects.^{7,11} Persistence of elevated urinary nitrite during remission indicates that nitric oxide might not be directly involved in the pathogenesis of nephrotic syndrome. Since all patients were receiving corticosteroids, it is probable that steroid therapy does not affect nitrite production. The persistently elevated nitrite excretion in relapse and remission suggested that renal nitric oxide synthesis does not modulate proteinuria. The clinical implication of these findings is that SSNS and SRNS are probably two distinct disorders with separate mechanisms for the development of glomerular dysfunction. At the onset of idiopathic nephrotic syndrome, it is difficult to distinguish between these two entities. The clinical response to corticosteroid therapy must be used to establish the diagnosis of SSNS versus SRNS. Measurement of urinary nitrite excretion, which is a simple and highly reproducible assay, may be a useful test to predict the response to steroid treatment. Steroid responsiveness is the single most important prognostic factor in idiopathic nephrotic syndrome. A lot of research has been done in this regard to find ideal parameter to know steroid responsiveness before starting steroid therapy. Urinary nitrite estimation appears to be simple and inexpensive test for the same. Some other parameters studied to predict steroid responsiveness are a) Selectivity index b) SDS PAGE and IEF (iso electric focusing) c) fractional excretion of magnesium d) IL-13 gene polymorphisms e) ACE gene I/D polymorphisms f) Ezrin- a estimation g) Myeloperoxidase enzyme assay.

All of these parameters are expensive to do and available only in very sophisticated labs. These are areas of interest in research but cannot be used in clinical practice routinely. The urinary nitrites as a predictor of steroid responsiveness have low sensitivity and low negative predictive value. Due to this one cannot avoid 8 weeks of steroid therapy in resistant cases based on negative urinary nitrite value. The high specificity and PPV may help to prognosticate children as steroid sensitive in cases of positive urinary nitrite value.

CONCLUSION

Urinary nitrite excretion is increased in children with steroid responsive nephrotic syndrome. This increase in urinary nitrite excretion is irrespective of the state of the disease. Steroid therapy has no effect on urinary nitrite excretion. Urinary nitrite excretion serves as a useful investigation in differentiating between steroid responsive and steroid resistant nephrotic syndrome. The urinary nitrite estimation has low sensitivity and high specificity in predicting steroid responsiveness.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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