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Biochemical evaluation of the possible role of nerve growth factor in female patients with Interstitial cystitis/Bladder Pain Syndrome

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ABSTRACT

Background:
interstitial cystitis/ Bladder pain syndrome (IC/BPS) is a chronic condition accompanied by lower urinary tract symptoms without evidence of urinary tract infection (UTI) last for at least 6 weeks. This condition affects women approximately five times more than men. The pathogenesis of the disease remains unclear Recently, IC/BPS has been classified into diffuse type and Ulcerative type with Honor's ulcer is no longer considered to be a part of BPS/IC. . Up till now, there is no specific biomarker for detection of IC/BPS.

Aim:
To evaluate the level of nerve growth factor (NGF) in the blood and urine in females with non- ulcerative interstitial cystitis compared to healthy individual females.

Subjects and methods:
This study included 92 female participants: 46 females with non-ulcerative interstitial cystitis and 25 age- matched healthy individual females were included as a control group, and 21 samples was taken from patients diagnosed to have overactive bladder as another group. All patients underwent thorough history and clinical examination. Urine analysis and urine cultures, abdominal and pelvic grey scale ultrasound were carried out. Cystoscopy was conducted for all patients. Disease severity was assessed using 3-day voiding diaries, the Likert visual analog scale (VAS) for pain, and the Interstitial Cystitis Symptom Index (ICSI) and the Interstitial Cystitis Problem Index (ICPI) of the O'Leary-Sant questionnaire. The level of serum and urine NGF was determined by ELISA technique. The possible co relation between NGF and patients' demographics, VAS, ICSI and ICPI was investigated.

Results:
Serum NGF was lower in IC/BPS compared with healthy control and OAB groups (P<0.05). While there was insignificant difference between healthy control and OAB groups. On the other hand, urine NGF was higher in IC/BPS compared with healthy control and OAB groups (P <0.5). While there was insignificant difference between healthy control and OAB groups. ROC analysis demonstrated that serum NGF is the best marker of the two studied markers in diagnosis of non- ulcerative interstitial cystitis (AUC of serum NGF is 0.744, p= 0.001).
Conclusion:
There is an increase in the level of NGF in urine and decrease in the level of NGF in serum of Patients with Non- ulcerative IC/BPS. These findings, might indicate the development of inflammation in this pathological condition in urine of patients. It could be a possible reliable marker for IC/PBS.

Keywords:
Nerve growth factor; Bladder pain syndrome; Interstitial cystitis; NHIC; ELISA.

Introduction

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic debilitating bladder disorder that causes lower urinary tract symptoms, such as suprapubic pain (pelvic pain; bladder pain), pressure, discomfort associated with filling the bladder, frequent urination and urgent urination without signs of a urinary tract infection. These symptoms must remain for at least six weeks (Doggweiler et al., 2017 and Homma et al., 2020). IC/ BPS is currently recognized as a distinguished type of heterogeneous syndrome named pelvic pain syndrome involved urinary bladder. There are two types of interstitial cystitis, Hunner interstitial cystitis (HIC) and non-Hunner interstitial cystitis (NHIC) based on the presence of Hunner lesions (Fall et al., 2014).

Physical, laboratory examination and cystoscopy are required for the clinical diagnosis of IC/BPS to document basic symptoms that characterize the disorder and exclude infections or other confusable disorders (Clemens et al., 2022). There is a need for a non-invasive marker for diagnosing the types of IC/BPS (Hann et al., 2012).

There is no definitive evidence to support an autoimmune, inflammatory, structural, or infectious etiology of IC/BPS (El-Hefnawy et al., 2015; El-Hefnawy et al., 2020). However, a central role of inflammation has been confirmed in human and animal studies (Sant et al., 2007). Persistent bladder inflammation damages urothelial integrity and increases urothelial barrier permeability (Jhang et al., 2018). In addition, central nervous sensitization and common neural hyperactivity may play a role in the pathophysiology of IC/BPS and functional somatic syndrome. Neuronal cross-talk with pelvic organs might exaggerate bladder symptoms (Warren et al., 2014).

Many studies have suggested that a number of neuropeptides (e.g. nerve growth factor (NGF) which activate submucosal afferent nerves and mast cells, are released by the urothelium (Theoharides et al., 2001). Since its discovery in 1950 has been associated with a broad range of pathological and physiological processes such as nerve development, growth and survival, inflammation and pain signaling (Masoudi et al., 2009). NGF enhancement appears to be a common feature of many other inflammatory diseases (Seidel et al., 2010). such as interstitial cystitis (Lowe

et al., 1997 and Jacobs et al., 2010). However, most of previous publications investigated heterogenous population with different entities of pelvic pain without sharp demarcations between ulcerative and non-ulcerative types. Moreover, data about concomitant evaluation of urinary and serum NGF in IC/PBS is still lacking in literature. This study aims to evaluate nerve growth factor level in non-ulcerative IC/BPS female patients and investigate the possible correlation between blood and urine NGF levels.

Subjects and Methods

The current study included 92 female participants: A total of 46 patients with non-ulcerative interstitial cystitis. In addition, 25 samples from the healthy individual females were included as a control group, and 21 samples was taken from patients diagnosed to have overactive bladder as control group. The proposal was submitted to the Institutional Research Board (MFM-IRB) in faculty of medicine, Mansoura University for the approval (ethical code: MS.21.03.1419.R1.R2).The consents were taken from the patients at Urology and Nephrology Center Mansoura University. Signed informed written consents from all subjects were obtained

Female Patients included in this work were diagnosed with non-ulcerative IC/BPS from the start of study onwards. The individuals excluded from the study if they had one of the following criteria: patients with ulcerative IC, patients with benign or malignant bladder tumors, radiation cystitis, cervical ulcers, history of urethral catheter, active herpes, tuberculous cystitis and patients receiving treatment for autoimmune disorders.

The patients' evaluation included, full history and clinical examination including brief neurological examination and gynecological examination. Urine analysis and urine culture for all patients were performed as apart of laboratory examinations. A grey scale ultrasound (US) on kidneys and urinary bladder with the full phase after the evacuation to detect post voiding residual urine (PVR) was conducted. A 3-day voiding diary was filled by patients. None invasive uroflowmetry (NIF) was carried out to assess the presence of obstruction. An outpatient cystoscopy was performed to confirm the diagnosis and determine the type of IC/BPS. Visual analogue scale for pain (VAS) was used to assessment severity of the condition. It ranges from 0 (least) to 10 (severest). Patients were asked to fill Interstitial Cystitis Symptom Index (4 questions) and Problem Index (ICSI/ICPI) (O'Leary et al., 1997).

Serum and urine samples were obtained from cases with non-ulcerative interstitial cystitis and from healthy bladder volunteers. Five ml of serum and urine samples were collected. Early morning, mid-stream urine samples were collected and urine was examined for pyuria that was defined by the presence of > 5 pus cells in the collected sample. Urine culture was carried out by blood agar technique. In case of suspicion of tuberculosis, a Zil Nelsen stain and polymerase chain reaction (PCR) for 3-day urine samples were carried out. Serum was collected for 10-15 minutes at room temperature and then centrifuged at 2000-3000 RPM for 20 minutes. Urine was collected by sterile tube and it was centrifuged at 2000-3000 RPM for 20 minutes then the supernatant was collected carefully.

Enzyme Linked Immunosorbent Assay (ELISA)

Serum and urine NGF levels were analyzed using an ELISA kit (Human Nerve Growth Factor ELISA Kit Cat.NoE2102Hu) purchased from Bioassay

Technology laboratory BT LAB, following the manufacturer's instructions, and the results are expressed in pg/ml. The plate has been pre-coated with human NGF antibody. NGF present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human NGF Antibody is added and binds to NGF in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated NGF antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of human NGF. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm using ELISA reader (chromate manager, USA).

Sample size and power analysis

Sample size was calculated by using Power Analysis and Sample Size (PASS) Software (version 15, 2017). NCSS, LLC. Kaysville, Utah, USA.

Hypothesis: We are expecting a considerable difference in serum NGF between the diseased cases (overactive bladder and IC) vs. healthy control with a large effect size, Cohen's f = 0.4). This was based on a previous study by Jacobs et al (Jacobs et al., 2010).

In a one-way ANOVA study, sample sizes of 20 healthy control, 20 overactive bladder, and 40 IC cases are obtained from the 3 groups whose means are to be compared. The total sample of 80 subjects achieves 89% power to detect differences among the means versus the alternative of equal means using an F test with a 0.0500 significance level. The size of the variation in the means is represented by the effect size $f = \sigma m / \sigma$, which is 0.4000.

Statistical analysis

Data were entered and analyzed using IBM-SPSS software (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Quantitative data were initially tested for normality using Shapiro-Wilk's test with data being normally distributed if $p>0.050$ and vice versa. Presence of significant outliers was tested for by examining boxplots. Quantitative data were expressed as mean ± standard deviation (SD) when normally-distributed, and median (interquartile range or range) when not normally-distributed. Qualitative data were expressed as count (percentage). One-Way ANOVA test was used to compare quantitative data between the three study groups when data are normally-distributed. Post-hoc analysis was used to properly compare each pair of data and results were expressed as letters. Games-Howell adjustment was used when assumption of equal variances are violated. Kruskal-Wallis H test was used to compare quantitative data between the three study groups when data are not normally-distributed. Pairwise comparison was used to properly compare each pair of data and results were also expressed as letters Independent sample t-test test was used to compare quantitative data between two groups when data are normally-distributed. Chi-Square test For any of the used tests, results were considered as statistically significant if p value ≤ 0.050. Bar charts, box plots and scatter plots were used to graphically present the results whenever needed.

Results

Demographic and laboratory parameters in the study groups:

There was no statistically significant difference in age, marital status, serum

creatinine between the three groups**.** Body mass index was higher in healthy control (p = 0.041) . The median VAS for pain in diseased group was 9.00 (range :7.00 – 10.00). For ICSI, median and range was 18.00 (14.00 -20.00) and according to Interstitial Cystitis Problem Index (ICPI), median and range was 14.00 (12.00 - 16.00) as shown in **(Table 1)**.

Assessment of serum and urine NGF in the three study groups

Serum NGF was lower in IC/BPS compared with healthy control and OAB groups (P<0.05). While there was insignificant difference between healthy control and OAB groups **(Figure 1, Table 2).** On the other hand, urine NGF was higher in IC/BPS compared with healthy control and OAB groups (P <0.5). While there was insignificant difference between healthy control and OAB groups. **(Figure 2, Table 2)**. ROC analysis demonstrated that serum NGF is the best marker of the two studied markers in diagnosis of non-ulcerative interstitial cystitis (AUC of serum NGF is 0.744, p= 0.001). **(Figures 3, 4 ; Table 3)**

Discussion

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a persistent condition that causes pain and discomfort in the bladder. The nature of IC/ BPS is not clear. The variety of symptoms of this disease shows that it has poly etiological origin (Patnaik et al., 2017).

The etiology of IC/BPS has been the subject of many different theories such as neuropathy, immunological theory, stagnation in the lymphatic system, influence of the infectious factors, corrosion of the mucous layer of the bladder, psychosomatic theory (formation of the disease against the background of psychological conditions), influence of toxins, etc. While none of these theories have been proven or fully refuted, but researchers thought that the last one would be the most possible: inflammation was caused due to seep toxic substance from urine through the mucous membrane to the bladder wall (Mullins et al., 2015; Lamb et al., 2017 and El Hefnawy et al., 2024). However, the precise reasons of this pathology's development are still unclear, due to the insufficient number of studies on it (Sholan, 2020).

The urothelium and bladder smooth muscle in the urinary tract produce NGF. The prevailing of data relate the effects of elevated or decreased NGF on bladder afferent fibers, despite the fact that NGF can affect a variety of cell types in the bladder. Data from both human and animals show that the bladder can increase production of NGF in response to a wide array of conditions including spinal cord injury, denervation, inflammation, distension or hypertrophy (Dupont et al., 2001).

In the current study, serum and urinary levels NGF was assessed by ELISA for all individuals participating in this study. Based on the results obtained in this study, the serum NGF was significantly decreased in non-ulcerative IC patients compared to healthy individual females.

Our findings are agreement with the finding of (Liu and Kuo, 2012) They suggest that other systemic illnesses may potentially be implicated in their condition and were not relate with more severe IC symptoms. Furthermore, there may be variations in the level of enhanced urothelial permeability in the IC/BPS bladder.

This disagree with the study of (Steers and Tuttle, 2006) which demonstrated that elevated serum NGF levels may lower the bladder dorsal root ganglia's excitatory threshold, increasing bladder wall's mechano-sensitivity. Thus, it is possible that levels of circulating serum NGF increase in response to changes in systemic conditions. This contrast may be due to medical co-morbidities rather than a cause of IC/BPS and may be due to this study carried out on non-ulcerative IC not ulcerative IC. Non Hunner interstitial cystitis may share a neurophysiological process with commonly known Functional Somatic Syndromes (FSSs) that cause central sensitization such as chronic fatigue syndrome, irritable bowel syndrome, and fibromyalgia (Bourke et al., 2015). Although the exact pathophysiology of FSSs is yet unknown, aberrant neuroimmune or endocrine processes with certain stressors have been suggested (Anderson et al., 2014).

The study of (Jablochkova et al., 2019) which demonstrated that the level of NGF was significantly lower ($p < 0.001$) in fibromyalgia than in healthy controls. The current study showed that the level of urine NGF was significantly increase in non-ulcerative IC patients compared to healthy control. This agrees with the study of (Kuo et al., 2010) which demonstrated that increased levels of NGF was also reported in the bladder tissue and urine of patients with sensory urgency and BPS/IC. Also, this agrees with the study of (Liu and Kuo, 2012) which demonstrated that increasing in urine NGF level predict that the patients might have chronic inflammation localized to the urinary bladder.

In addition, this agrees with the study of (lin et al., 2022) which demonstrated that NGF is associated with bladder function and that higher urine NGF levels indicate chronic inflammation bladder of patients with IC/BPS. Patients with severe urothelial leakage may have a higher urine NGF level without a measurable bladder NGF level.

ROC curve analysis of serum NGF in the diagnosis of non -ulcerative interstitial cystitis shows a predictive power, as demonstrated by the area under the curve, of 0.744 ($P<0.001$). This indicates that serum NGF analysis by ELISA may be suitable for diagnosis of non–ulcerative interstitial cystitis with 57% sensitivity and 94% specificity at cut off $\leq$ 8.16 pg/ml.

ROC curve analysis of urine NGF in the diagnosis of non -ulcerative interstitial cystitis shows a predictive power, as demonstrated by the area under the curve, of 0.573 ($P=0.226$) with 59% sensitivity and 67% specificity at cut off $\geq$ 2.19 pg/ml. This indicates that urine NGF analysis by ELISA may be unsuitable for diagnosis of non–ulcerative interstitial cystitis.

On the other hand, results showed that NGF was lower in patients with overactive bladder. This could be explained by the fact that NGF as a neurotrophic factor has many biological functions including promotion and protection of the nerve growth (Lewin and Barde, 1996**).** This function might be deficient in OAB patients and could be a possible underlaying mechanism of development of the

disease. Since one of the differential diagnosis of IC/BPS is OAB, NGF could be a suitable marker with high specificity in such case.

Conclusion

There is an increase in the level of NGF in urine and decrease in the level of NGF in serum of Patients with Non- ulcerative IC/BPS. These findings, might indicate the development of inflammation in this pathological condition in urine of patients. It could be a possible reliable marker for IC/PBS.

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Table 1: Demographic and laboratory parameters in the three study groups:

Variable	Group			Test statistic	p value	Partial η2
	Healthy control (n=25)*	**Overactive bladder (n=21)***	**Non-ulcerative IC (n=46)***			
Age (years)	37.89 ± 10.65	37.71 ± 8.66	36.42 ± 3.72	F= 0.263	0.770#	0.006
Body mass index (kg/m2)	29.29 ± 2.39 A	26.94 ± 2.86 B	27.24 ± 4.07 B	F= 3.332	**0.041#**	0.074
Marital status: Single: Married: Divorced**:** *Missed data:	 0 (0%) 24 (96%) 0 (0%) 1 (4%)	 0 (0%) 18 (85.7%) 0 (0%) 3 (14.3%)	 2 (4.3%) 42 (91.3%) 1 (2.2%) 1 (2.2%)	χ^2-= 7.154	0.307**	-
Serum creatinine (mg/dl)	0.87 ± 0.03	0.87 ± 0.02	0.85 ± 0.02	F= 0.282	0.755#	0.007

*Data are presented as mean ± standard deviation or count (percent). #p value by One-Way ANOVA, pairwise comparison using Games-Howell adjustment, post-hoc test is presented as capital letters (similar letters = statistically insignificant difference, different letters = statistically significant difference), bold values indicate significant p values (≤0.05). ** p value by Chi-Square Test.*

Table 2: Comparison of serum and urine NGF in the three study groups:

Parameters	Groups			H	P value
	Healthy control (n=25)	**Overactive bladder (n=21)**	**Non-ulcerative IC (n=46)**		
Serum NGF concentration (pg/ml)	27.54 (17.10 – 56.12) A	27.33 (8.58 – 62.92) A	6.99 (3.50 – 23.89) B	16.775	**<0.001**
Urine NGF concentration (pg/ml)	9.32 (5.545 – 20.02) A	20.98 (12.39 – 43.75) A	28.18 (6.73 – 49.23) B	6.199	**0.045**

Data are presented as median (interquartile range). p value by Kruskal-Wallis H test, Pairwise comparisons are presented as capital letters (similar letters = statistically insignificant difference, different letters = statistically significant difference), bold values indicate significant p values (≤0.05).NGF: nerve growth factor.

Table 3: ROC curve analysis of the serum and urine NGF in diagnosis of non-ulcerative interstitial cystitis:

Diagnostic marker	AUC (95% CI)	SE	P	SN	SP	PPV	NPV
Serum NGF At cutoff value of (≤8.16 pg/ml)	**0.744 (0.636 – 0.851)**	**0.055**	**<0.001**	**57%**	**94%**	**89.7%**	**68.8%**
Urine NGF At cutoff value of (≥21.19 pg/ml)	**0.573 (0.453 – 0.694)**	**0.061**	**0.226**	**59%**	**67%**	**62.8%**	**62%**

AUC: Area under the ROC curve; 95% CI: 95% confidence interval; SE: Standard error; PPV: Positive predictive value; NPV: Negative predictive value; Bold values: significant p

value (≤ 0.050).

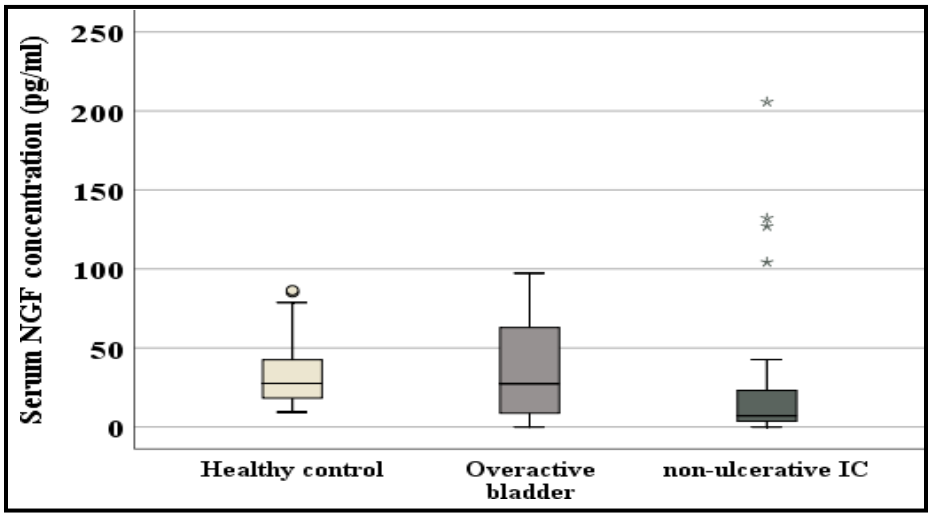

Figure (1): Comparison of serum NGF in the three study groups

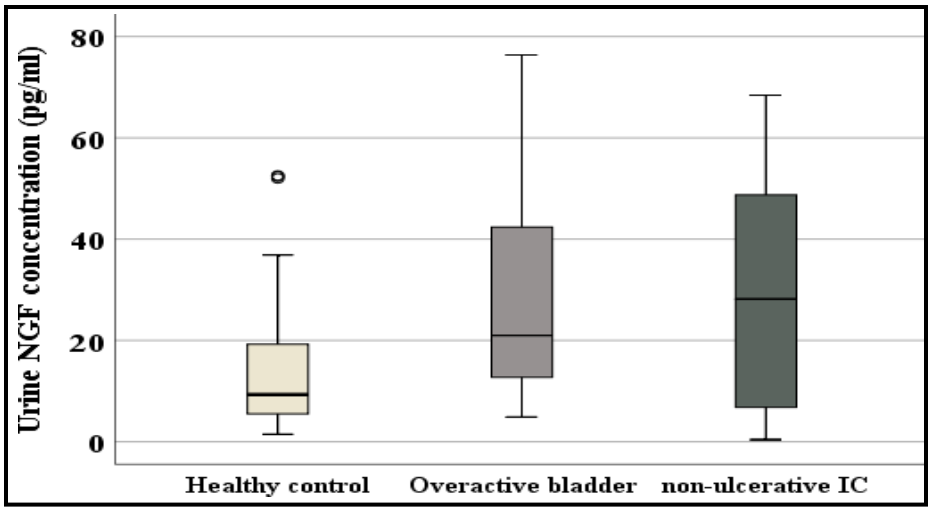

Figure (2): Comparison of urine NGF in the three study groups

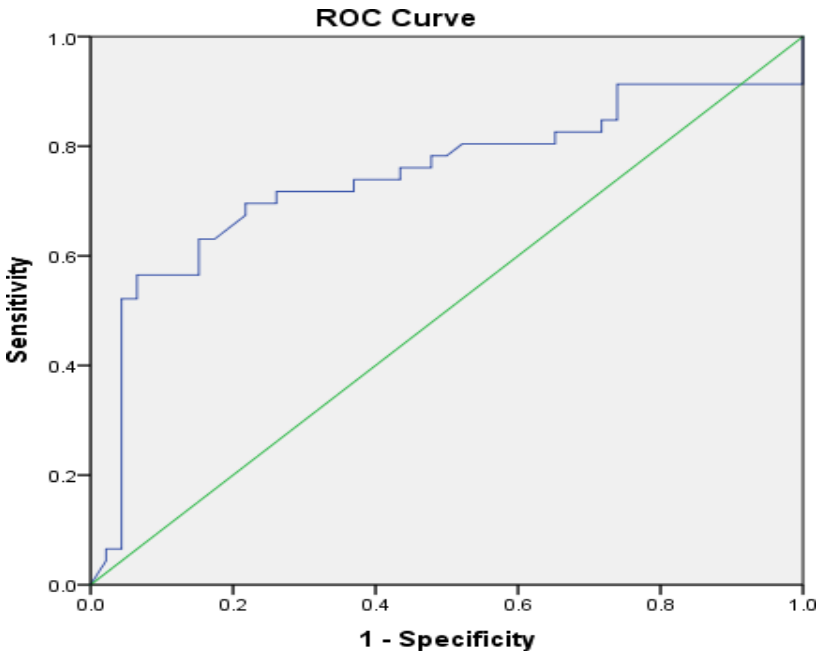

Figure (3): ROC curve of serum NGF in diagnosis of non-ulcerative interstitial cystitis.

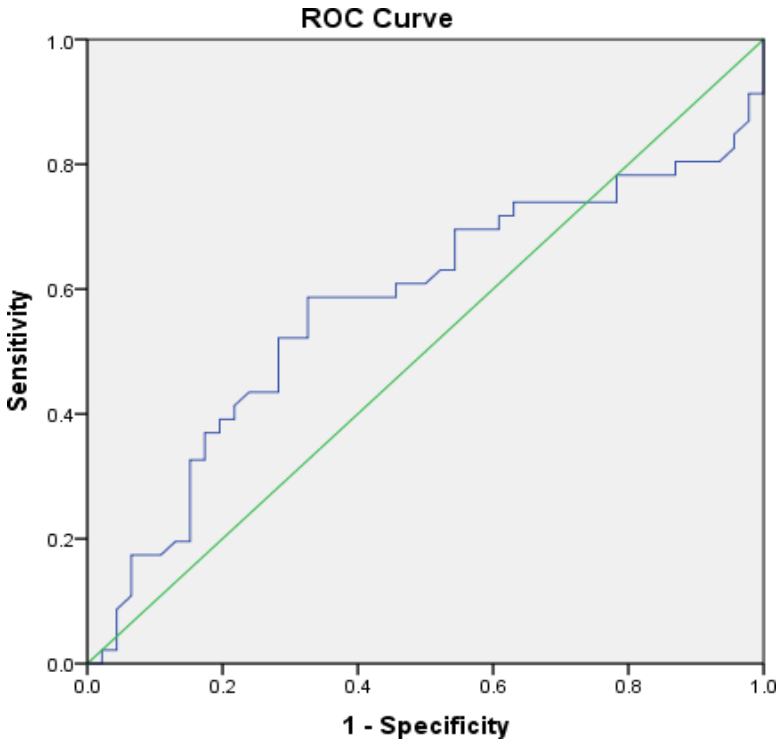

Figure (4): ROC curve of urine NGF in diagnosis of non-ulcerative interstitial cystitis