Can Urinary Nerve Growth Factor and Brain-Derived Neurotrophic Factor be used in the Diagnosis and Follow-Up of Voiding Dysfunction in Children?

Kadriye Ozdemir,[1]* Nida Dincel[2] Afig Berdeli,[3] Sevgi Mir[1]

Purpose: We investigated the utility of urinary nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) levels as non-invasive markers for diagnosis and evaluation of treatment efficacy in children with overactive bladder (OAB).

Materials and Methods: This prospective study included 24 children with OAB and 30 healthy controls. At the time of diagnosis, micturition disorder symptom scores (MDSS) were determined, blood and urine samples were collected, and anticholinergic therapy was initiated. Clinical responses were evaluated, at the third and sixth month of treatment, by MDSS and urinary NGF, BDNF, and creatinine levels.

Results: The patient group had significantly higher urine NGF/Cr ratio (975 ± 827 and 159 ± 84, respectively, $P < .001$) and BDNF/Cr ratio (5.98 ± 5.78 and 0.81 ± 0.70, respectively, $P < .001$) before treatment. Significantly decreased BDNF/Cr ratio was found at the sixth month (5.98 ± 5.78 and 2.24 ± 0.98, respectively, $P = .004$). NGF/Cr > 360 was found to have 87.5% sensitivity and 100% specificity, and BDNF/Cr > 1.288 was found to have 87.5% sensitivity and 83.3% specificity for OAB diagnosis.

Conclusion: In conclusion, urine NGF/Cr and BDNF/Cr ratios may be useful markers for diagnosis of OAB. The BDNF/Cr ratio was found to be more significant in monitoring treatment response.

Keywords: biomarkers/urine; case-control studies; nerve growth factor; urinary bladder/physiopathology; urinary bladder, overactive/etiology.

INTRODUCTION

Bladder dysfunction (BD) is a common problem in childhood, presenting with lower urinary tract symptoms including weak urine stream, frequency, urgency, urge incontinence, and urinary tract infections (UTI). Potential causes of BD were hypothesized as delay in neurologic maturation and anatomical and neurological abnormalities. Mistakes in potty training or disturbances during toilet training may cause BD symptoms in children.[(1)] More attention has been paid on cases of overactive bladder (OAB). Among BD cases, children with OAB deserve more attention. OAB is commonly seen between 5 and 7 years of age, and is one of the most common causes of functional urinary incontinence in children.[(2)] The diagnosis of OAB is made based on urgency, with or without urge incontinence, usually with voiding frequency and nocturia, in the absence of an underlying metabolic or pathological condition.[(3)]

Use of non-invasive methods including clinical symptom scores, micturition diaries, micturition symptom scoring systems, and post-void residual urine measurements can be helpful in the diagnosis of OAB. To confirm the diagnosis of OAB, detrusor instability should be detected on urodynamic measurements. However, urodynamic study is an invasive method, and many external factors, including alterations in mental situation, hydration status, and the disease process itself may affect study results.[(4)] Thus, in children it is difficult to perform urodynamic measurements. Some studies have suggested a weak correlation between clinical symptoms and urodynamic findings in patients with OAB.[(4)] Therefore, readily applicable non invasive objective determinants are required for the diagnosis of OAB in pediatric age group. Urinary cytokines, C-reactive protein (CRP), prostaglandins, and detrusor wall thickness have been suggested as markers for the diagnosis of OAB. However, none of these measurements enter into routine clinical practice.[(5,6)]

The role of neurotrophic factors was shown in bladder development and function and in the micturition pathway.[(7)] Nerve growth factor (NGF) was the first mem-

[1] Department of Pediatric Nephrology, Ege University School of Medicine, İzmir 3500, Turkey.
[2] Department of Pediatric Nephrology, Pediatric Hematology Oncology Training and Research Hospital, Ankara 0600, Turkey.
[3] Department of Molecular Biology, Ege University School of Medicine, İzmir 3500, Turkey.
*Correspondence: Department of Pediatric Nephrology, Ege University School of Medicine, İzmir 3500, Turkey.
Tel: +90 232 4116281. Fax: +90 232 390 10 84. E-mail: kcanturk1@hotmail.com.
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ber of the neurotrophin (NT) family discovered; other neurotrophins include brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5.[8,9]

NGF is the most frequently investigated neurotrophin in adults.[10] NGF is produced in the bladder epithelium and smooth muscle cells. Increased NGF expression has been detected in situations such as detrusor over-activity, interstitial cystitis/painful bladder syndrome (IC/PBS), and overactive bladder syndrome.[11] Recent studies have shown increased urine NGF levels in adults with OAB, and NGF may be a potential indicator of OAB.[12] To our knowledge, up to date, only one study has been conducted in children related to this condition.[13]

Another neurotrophin, BDNF, is the most prevalent but least studied in the body. Clinical studies on adults have documented significantly increased urine BDNF levels in patients with OAB compared to healthy controls.[14] Furthermore, enzyme-linked immunosorbent assay (ELISA) yielded higher sensitivity and specificity in the detection of BDNF than that of NGF.[15] According to our English literature search, no study investigating BDNF levels in children with OAB has been reported. We hypothesized that NGF and BDNF may be elevated in children with OAB, which would be valuable to diagnose OAB, and levels of NGF and BDNF may be correlated with symptoms. The aim of this study was to investigate the utility of urine NGF and BDNF levels as diagnostic markers and as biomarkers during follow-up in children with OAB.

MATERIALS AND METHODS

Study Population

The study group consisted of 24 children aged 5-15 years who had been diagnosed with first-onset voiding dysfunction at the Pediatric Nephrology Clinics of Ege University. The control group included 30 healthy, age-matched children with no symptoms of lower urinary system dysfunction, no history of a disease, symptom, or sign indicating UTI, and no urgency or urge incontinence. They were chosen from children who applied to our hospital for routine check-up or elective surgery, such as hernia repair or circumcision, who had no nephro–urological or neurological dysfunction or lower urinary tract symptoms. Ethical approval for the study was obtained from the Clinical Research Ethics Committee of Ege University School of Medicine. Informed consent was obtained from children and/or their parents before initiation of the study.

Procedures

Urodynamic studies were done using an urodynamic instrument (Aymed DYNO Urodynamics, Istanbul, Turkey). The same nurse, accompanied by a physician specialist in pediatric nephrology, performed urodynamic studies and the results were evaluated by the same specialist. After a diagnosis was made, anticholinergic therapy with oxybutynin at a total dose of 0.3-0.5 mg/kg/day divided into three doses was started. No urodynamic study was performed in the control subjects.

Urine samples were collected before the initiation of the therapy and at the third and sixth months, and treatment response was evaluated by MDSS. Urine samples of 5 mL were obtained in the morning at room temperature, and stored at -80°C until measurements. Another 3 mL urine was stored separately to measure creatinine levels.[12] Urine samples were obtained as free-voided samples without catheterization in all children.

Quantitative urinary NGF measurements were done using a human beta-NGF ELISA kit (ab99986 beta-NGF Human ELISA Kit, ABCAM, Cambridge, UK). Assay sensitivity was < 14 pg/mL and the detection range was 6.86–5000 pg/mL. Quantitative BDNF measurement in urine was performed using a human BDNF ELISA kit (ab99978 BDNF Human ELISA Kit, ABCAM). Assay sensitivity was < 80 pg/mL and the detection range was 0.066–16 pg/mL.

For the quantification of NGF and BDNF levels, the urine samples were diluted and tested as indicated in the manufacturer's protocols. Briefly, after the preparation of NGF and BDNF reactive ingredients, samples and standards, 100 µL of the standard or samples was added to the wells and incubated for 2.5 h at room temperature (RT). Then, 100 µL of biotin antibody solution was added to the wells and incubated for 1 h. After incubation, 100 µL of streptavidin solution was added and incubated for about 45 min at RT. Careful washing was carried out between steps. Then, tetra methyl benzidine (TMB) reactive substrate was added to the wells and incubated for about 30 min until the color development was visualized. Stop solution was then added and the wells were read at 450 nm with an ELISA reader.

Each sample was tested twice using ELISA and the mean value was obtained. Measured urine NGF (pg/mL) and BDNF (ng/mL) levels were divided by urine creatinine (Cr, mg/dL) for standardization as NGF/Cr and BDNF/Cr ratios.

Evaluations

In the urodynamic study, the diagnosis of bladder instability was made by taking into account the relaxed condition of the pelvic floor muscles during the micturition phase along with uncontrolled detrusor contractions. Detrusor over activity was confirmed by the existence

Table 1. Demographic characteristics of the patients and the controls and urinary NGF/Cr and BDNF/Cr levels.*

Variables	Patients (n = 24)	Controls (n = 30)	*P* Value
Boy/Girl, no	6/18	8/22	.890
Age, month	109.7 ± 31.8	110.7 ± 31.9	.915
Weight, kg	30.8 ± 13.8	32.7 ± 15.7	.652
Height, cm	132.4 ± 16.6	133.6 ± 17.1	.797
Urine NGF/Cr ratio	975 ± 827	159 ± 84	< .001
Urine BDNF/Cr ratio	5.98 ± 5.78	0.81 ± 0.70	< .001

Abbreviations: NGF, Nerve Growth Factor; BDNF, Brain Derived Neurotrophic Factor, Cr: Creatinine.
* Data are presented as mean ± SD.

of uncontrolled contractions of the detrusor muscle during the filling phase in the urodynamic study.

All participants and their accompanying family members were asked to complete the Micturition Disorder Symptom Scoring (MDSS) questionnaire developed by Akbal and colleagues,[16] which consisted of 13 questions (scores of 0-35). A decrease in MDSS score was considered a measure of clinical treatment response.

Inclusion and Exclusion Criteria

Inclusion criteria: Patients with OAB who exhibited one or more symptoms meeting the diagnostic criteria for voiding dysfunction, including the need to urinate at frequent intervals daily (urinating often but in lower volumes), feeling that the bladder is not completely empty were included. Incontinence during the daytime, a feeling of sudden pressure, maneuvers such as crossing the legs to hold back urination until reaching the bathroom, dripping urine, dysuria (painful urination), straining during urination and intermittent flow during urination were considered other findings of voiding dysfunction and these patients also were included.

Exclusion criteria: Patients with current or recent UTI were excluded. Children with functional or anatomical bladder outlet obstruction and those with findings of neurological dysfunction were also excluded.

Statistical Analysis

A power analysis was performed considering the results of a previous study.[17] We calculated a sample size of 24 patients in the study group and 30 subjects in the healthy control group for $\alpha = 0.05$ and $\beta = 0.20$ with a power of 0.90 in a two-tailed test. The Statistical Package for the Social Science (SPSS Inc, Chicago, Illinois, USA) version 18.0 was used for statistical analyses. The normality of the distribution of quantitative variables was tested using the Kolmogorov-Smirnov test. Student's *t*-test was used to compare two groups on variables satisfying a normal distribution, and the Mann–Whitney *U* test was used to compare data that was not normally distributed or that was derived from a few individuals. For repeated measures of the same group, a paired *t*-test or Wilcoxon test was used. Pearson's or Spearman's correlation analyses were used to evaluate the relationships between quantitative variables. The χ^2 test was used to compare categorical data. Cut-off values, along with sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), were determined for NGF/Cr and BDNF/Cr using a receiver operating characteristic (ROC) curve analysis. A P value of less than .05 was considered statistically significant.

RESULTS

In total, 24 pediatric patients with urinary dysfunction (6 boys, 18 girls; age range 5-15 years, mean age 109.7 ± 31.8 months), and 30 gender- and age-matched healthy control children (8 boys, 22 girls) with no overt urinary dysfunction were included in the study. Patient symptoms at the first visit were frequency (n = 13), micturition with intermittent flow (n = 9), constipation (n = 8), urgency (n = 24), enuresis nocturnal (n = 21), urinary incontinence (n = 20), and recurrent UTI (n = 10). An urodynamic study was performed, and detrusor over activity was detected in all patients. Patients had significantly higher urine NGF/Cr (975 ± 827 vs. 159 ± 84; $P < .001$) and BDNF/Cr ratios (5.98 ± 5.78 vs. 0.81 ± 0.70; $P < .001$) at the first (before treatment) measurement compared with the healthy controls (**Table 1**). In the patient group, MDSS and urine ratios of NGF/Cr and BDNF/Cr before (first measurement) and at 3 and 6 months after treatment were compared. MDSS scores decreased gradually from the first measurement to the second and third measurements. The mean MDSS scores were 24, 11.1, and 6.4 at the first, second, and third measurements, respectively. Significant differences were found in MDSS scores between the first measurement and that at 3 months, between the first measurement and that at 6 months, and between

Table 2. Comparison of the measurement in patient group pre-treatment, at 3rd month and 6th month time points.*

Variables	Before Treatment a	3rd Month b	6th Month c	*P* Value
MDSS	24.0 ± 6.1	11.1 ± 6.7	6.4 ± 6.7	$^{a-b}$< .001, $^{a-c}$ < .001, $^{b-c}$ .002
Urine NGF/Cr	975 ± 827	660 ± 353	723 ± 435	$^{a-b}$ .049, $^{a-c}$ .097, $^{b-c}$ .531
Urine BDNF/Cr	5.98 ± 5.78	2.17 ± 1.37	2.24 ± 0.98	$^{a-b}$ .005, $^{a-c}$ .004, $^{b-c}$ .831

Abbreviations: MDSS, Micturition Disorder Symptom Scoring; NGF, Nerve Growth Factor; BDNF, Brain Derived Neurotrophic Factor; Cr, Creatinine.
* Data are presented as mean ± SD.

the third- and sixth-month measurements ($P < .001$, $P < .001$, and $P = .002$, respectively; **Table 2**).

When NGF/Cr ratios were compared among the three measurements, although there was a slight decrease at the third month and some increase at the sixth month, only a marginally significant difference was found between the first and third month measurements ($P = .049$). However, no significant difference was detected between the first measurement and that at month 6 or between the third- and sixth-month measurements ($P > .05$; **Table 2**).

Comparison of the urine BDNF/Cr ratios among the first, second, and third measurements showed significant decreases between the first (before treatment) and second (third month) measurement ($P = .005$) and between the first and third (sixth month) measurement s ($P = .004$), but there was no significant difference between third- and sixth-month measurements ($P = .831$; **Table 2**).

Correlation analyses among MDSS and ratios of NGF/Cr and BDNF/Cr showed significant correlations. Positive correlations were found between MDSS and NGF/Cr at the first measurement ($r = .650$, $P = .001$), between MDSS and BDNF/Cr at the third month ($r = .443$, $P = .049$), and between NGF/Cr and BDNF/Cr at all three measurement points ($r = .713$, $P < .001$; $r = .550$, $P = .010$; and r = .443, $P = .035$, respectively).

Considering the pre-treatment (first measurement) levels of NGF/Cr and BDNF/Cr ratios, values of NGF/Cr and BDNF/Cr were calculated to determine the cut-off point for designating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). At a cut-off level of NGF/Cr > 360, the sensitivity was 87.5%, specificity 100%, PPV 100%, and NPV 90.9%. At a cut-off level of BDNF > 1.288, the sensitivity, specificity, PPV, and NPV values were 87.5%, 83.3%, 80.8%, and 89.3%, respectively (**Table 3**). The ROC curve for the first measurements of NGF/Cr and BDNF/Cr is shown in **Figure**.

DISCUSSION

It is difficult to make a diagnosis and detect disease severity by medical history or symptoms in children with voiding dysfunction. To evaluate treatment efficacy, it is important to be able to determine changes in the severity of clinical symptoms. To make clinical evaluations more objectively, symptom-scoring systems have been developed. In the current study, we used the MDSS, developed by Akbal and colleagues[16] that detected the MDSS cut-off value of 8.5, and the test was shown to have sensitivity and specificity of 90%.[16] Before treatment, we determined the scores of MDSS to be > 9 in all of our 24 patients. We observed gradually decreasing MDSS values during the treatment process, supporting the position that MDSS may be used as a clinical measure in the monitoring of treatment responses in voiding dysfunction. In our study, urine NGF and BDNF levels were tested pre-treatment and twice after treatment during follow-up. These values were evaluated together with MDSS scores in OAB children by clinical and urodynamic examinations.

It is known that, NGF is secreted from the bladder epithelium and smooth muscle cells in the urinary system, and is detected in high levels in some conditions, such as detrusor over activity, IC/PBS, and OAB.[18] In two previous studies, significantly higher NGF and prostaglandin levels were found in adult male and female patients with OAB versus healthy controls.[19] In the study

Table 3. Urine NGF/Cr and BDNF/Cr cut-off values and their sensitivity, specificity, positive predictive value and negative predictive values.

Cut-off Value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
NGF/Cr > 360	87.5	100	100	90.9
BDNF/Cr > 1.288	87.5	83.3	80.8	89.3

Abbreviations: PPV, Positive Predictive Value; NPV, Negative Predictive Value; NGF, Nerve Growth Factor; BDNF, Brain Derived Neurotrophic Factor; Cr, Creatinine.

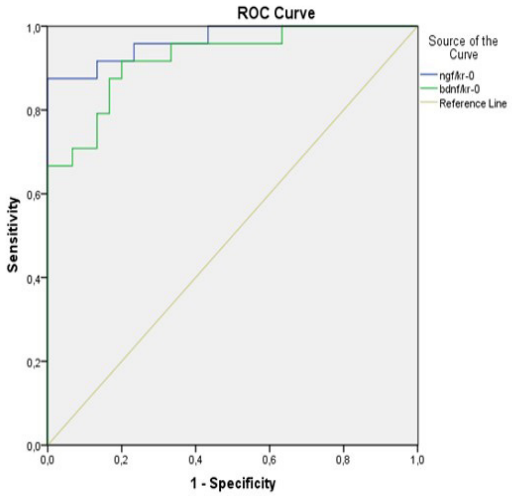

Figure. ROC curve for NGF/Cr vs. BDNF/Cr values.
Abbreviations: ROC, receiver operating characteristic; NGF, Nerve Growth Factor; BDNF, Brain Derived Neurotrophic Factor; Cr, Creatinine.

by Liu and colleagues,[20] significantly elevated levels of urinary NGF/Cr were detected in patients, and NGF/Cr levels decreased markedly in 50 responsive patients at the third month after treatment, but there was no decrease in 20 unresponsive patients. In the same study, a decrease in urine NGF levels was reported, along with attenuation in urgency, and an increase in urine NGF levels was seen with emerging OAB symptoms.

Although many studies evaluating NGF levels in adult patients with OAB have been conducted,[19] there are few studies with children. In the study by Oktar and colleagues, urine NGF and NGF/Cr levels were measured in 40 pediatric patients with OAB and 20 healthy controls.[13] They reported higher NGF levels and higher NGF/Cr ratios in the patients before antimuscarinic therapy compared with the healthy controls, with a significant decrease at the sixth month after therapy.[13] In our study, significantly higher NGF/Cr levels were detected in 24 pediatric patients with OAB compared with the controls before treatment. At the third month, a distinct decrease was observed, but an elevation was seen again at the sixth month.

Although the sensitivity and specificity of urine NGF/Cr have been reported in adult studies in the literature, no data exist regarding children. In a recent study, the sensitivity and specificity of urine NGF/Cr were reported as 61.9% and 68.4%, respectively, in the diagnosis of OAB in women,[15] whereas another study on adult patients reported 54.4% sensitivity and 95.6% specificity for NGF/Cr in OAB diagnoses.[21] In our study, a urine level of NGF/Cr > 360 was found to have 87.5% sensitivity for OAB diagnosis and 100% ability to correctly distinguish healthy individuals. Our results indicate that NGF/Cr levels had considerably higher sensitivity and specificity in the diagnosis of OAB in children than has been suggested by results in adults.

When we evaluated the clinical importance of urine NGF/Cr levels related to treatment response monitoring, we detected significant attenuation in response at the third month. Only the difference in measurements between the first and third month was statistically significant; no statistically significant difference was detected between the remaining measurements. In addition, although significant gradual decreases in patients' MDSS scores were recorded at the third- and sixth-month evaluations compared with the initial measurements, indicating a positive treatment response, the lack of parallel decreases in NGF/Cr levels indicates that NGF/Cr levels may not be a good marker for monitoring treatment responses. In the study by Oktar and colleagues, markedly attenuated NGF levels were reported at the sixth month after treatment.[13] In our patient group, although a significant decrease in urine NGF/Cr level was seen at the third month, the level at the sixth month was indistinguishable from that of before treatment. Also, the correlation of MDSS with the NGF/Cr level alone before treatment combined with the disappearance of this correlation during treatment suggest that NGF/Cr is not a reliable parameter for monitoring treatment response.

BDNF is expressed in inflamed bladder tissue,[22] and its expression in the bladder increases dramatically during chronic cystitis and following spinal cord injury.[23] Recent studies have shown that urinary BDNF levels are high in patients with interstitial cystitis/painful bladder syndrome U (IC/PBS) and diminish after botulinum toxin injection.[24] In those patients, pain attenuation accompanied the lowered results for BDNF.

In comparison with healthy controls, patients with OAB have increased levels of urinary BDNF, and a decrease was recorded in BDNF levels following behavior modification and antimuscarinic therapy.[14,25] Pinto and colleagues stated that intravenous delivery of a recombinant protein that neutralizes BDNF activity, diminished bladder contractions in rats with chronic cystitis, suggesting BDNF activity has a pivotal role in bladder function.[26] Similar to NGF, higher urinary BDNF concentrations were also found in patients with IC/PBS, and the level significantly decreased after botulinum injections.[24] In our study, urinary BDNF/Cr levels were significantly higher in patients than in healthy controls and the urine cut-off level of BDNF/Cr > 1.288 was determined to have high sensitivity and specificity for

OAB diagnosis. Thus, we thought that similar to NGF/Cr levels, the BDNF/Cr levels also useful parameter for OAB diagnosis.

This study is the first to evaluate the sensitivity and specificity of NGF/Cr and BDNF/Cr in children with OAB. In one study on adult patients with OAB, BDNF/Cr was reported to have 71.4% sensitivity and 89.5% specificity;[15] in another study, the sensitivity and specificity were found as 88.9% and 93.3%, respectively.[21] With regard to the diagnosis of OAB, although the sensitivity of NGF/Cr and BDNF/Cr was at the same level in the present study, the specificity of NGF/Cr was higher. In clinical evaluations, a distinct decrease was observed in MDSS from the first measurement to the third measurement at sixth month. There was also a decrease in BDNF/Cr levels at the third and sixth months compared with the first measurement. The only correlation between MDSS and BDNF/Cr was seen at the third month. Given this, BDNF/Cr seems to be a better parameter than NGF/Cr for monitoring the treatment response. In a study by Antunes and colleagues, NGF/Cr and BDNF/Cr levels in urine samples of 21 female patients with OAB were shown to decrease significantly following treatment.[15] In our study, BDNF/Cr levels were diminished at the third month, and the low levels were preserved at the sixth month, whereas NGF/Cr levels were significantly lower only at the third month. In a clinical study conducted by Wang and colleagues, a positive correlation was found between a decrease in symptom scores and NGF/Cr and BDNF/Cr levels.[21] In a study of the relationship between the Indevus Urgency Severity Scale (IUSS), a scoring system that measures the severity of urgency and urinary dysfunction, and levels of NGF/Cr and BDNF/Cr, the only association was between BDNF/Cr and IUSS.[15,21] In the current study, we found positive correlations between NGF/Cr and MDSS at the diagnosis and between BDNF/Cr and MDSS at the third month. We suggest that even though BDNF/Cr levels decreased significantly and gradually, the absence of a correlation between BDNF/Cr and MDSS, except at the third month, may be related to the small patient population in this study. The results of this study can only be applied to pediatric age group, because we included only children. Another limitation of the study is the lack of follow-up for NGF and BDF measurements at the third and sixth months in healthy controls for comparison with the patients.

CONCLUSIONS

In conclusion, we evaluated the clinical significance of NGF and BDNF in OAB diagnosis and in monitoring treatment responses in children with OAB. We showed that urinary NGF/Cr could be a useful marker for OAB diagnosis, and BDNF/Cr may be a beneficial marker for evaluating treatment efficacy in children with OAB.

CONFLICT OF INTEREST

None declared.

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