

**Title Page****Title:**

Evaluation of urinary aquaporin-2 and plasma copeptin as biomarkers of effectiveness of desmopressin acetate for the treatment of monosymptomatic nocturnal enuresis

**Authors:**

Taichi Hara, Yoshiyuki Ohtomo\*, Amane Endo, Shinichi Nijima, Masato Yasui and Toshiaki Shimizu

**Affiliations:**

From the Department of Pediatrics and Adolescent Medicine, Juntendo University Graduate School of Medicine (TH, AE, TS), Department of Pediatrics Juntendo University Nerima Hospital (YO, SN), and Department of Pharmacology, Keio University School of Medicine (MY), Japan

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**Corresponding author:**

\*Yoshiyuki Ohtomo, MD, PhD

Department of Pediatrics

Juntendo University Nerima Hospital

3-1-10 Takanodai, Nerima-ku, Tokyo 177-0033, Japan

Tel: (81) 3 5923 3111 (ext.) 6023

Fax: (81) 3 5923 3217

E-mail: ohtomo@juntendo-nerima.jp

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## **Abstract**

**Purpose:** Desmopressin is a synthetic V2-specific analog of antidiuretic hormone (arginine vasopressin), widely used as a first-line treatment for monosymptomatic nocturnal enuresis. However, no biomarkers to predict desmopressin effectiveness have yet been established. Because arginine vasopressin is unstable, we prospectively measured the major urine concentration factors aquaporin-2 and copeptin (as a surrogate marker for vasopressin) in patients with monosymptomatic nocturnal enuresis, and evaluated whether they are useful for predicting desmopressin treatment outcome.

**Materials and Methods:** Thirty-two children (aged 6–11 years) with monosymptomatic nocturnal enuresis and nocturnal polyuria were included. Exclusion criteria were daytime urinary symptoms and underlying diseases causing nocturnal enuresis. Subjects were treated with desmopressin orally disintegrating tablet (120/240 µg) and divided into desmopressin responders (grouped by effective dose: 120R/240R) and non-responders (240NR). Day/night ratios of plasma copeptin and urinary aquaporin-2 were measured during desmopressin treatment.

**Results:** There was no significant difference in baseline day/night ratio of urinary aquaporin-2 between desmopressin responders and non-responders. After 8 weeks' treatment, there was a significant correlation between day/night ratio of aquaporin-2 and percentage of wet nights. In responders, but not non-responders, there was a significant difference in the change in aquaporin-2 day/night ratio from before treatment to complete remission ( $p=0.0004$ ). For plasma copeptin, the baseline day/night ratio in 120R was significantly lower than in 240NR ( $p=0.02$ ).

**Conclusions:** Urinary aquaporin-2 appears to be a biomarker of desmopressin treatment effectiveness during treatment, while plasma copeptin levels before treatment are predictive of subsequent desmopressin response.

## **Introduction**

Approximately 50% of patients with nocturnal enuresis have nocturnal polyuria, for which first-line pharmacological treatment is 1-desamino-8-arginine vasopressin (dDAVP), a selective vasopressin V2 receptor agonist<sup>1</sup>. The mechanisms by which dDAVP reduces enuresis have not been fully elucidated, but it has been demonstrated to decrease urinary output in hydrated children with PN<sup>2</sup>. Because no reliable biomarkers of dDAVP treatment effectiveness have yet been established, frequency-volume charts or bladder diaries are routinely used to guide diagnosis and treatment selection according to urinary volumes<sup>1</sup>.

Arginine vasopressin (AVP) and aquaporin-2 (AQP2) are well recognized as major determining factors for urine concentration<sup>3</sup>. AVP causes the kidneys to conserve water, leading to an increased concentration and reduced volume of urine<sup>4</sup>, and AQP2 reflects the AVP-mediated urine concentrating mechanism<sup>3</sup>. However, instability of AVP makes reliable measurement of the hormone difficult and precludes routine use as a predictor of treatment success. Over 90% of AVP in the circulation is bound to platelets and rapidly cleared, resulting in underestimation of AVP levels<sup>5</sup>. Circadian rhythm of AVP secretion was first described by George et al.<sup>6</sup>, who observed a nocturnal increase in AVP in healthy adult males and a nocturnal decrease in urine production to around half of daytime volumes. Clinical analysis of AVP measurements shows that NE patients have a blunted diurnal rhythm of AVP secretion. This was later confirmed by several investigators,<sup>7,8</sup> but not by Eggert et al.<sup>9</sup>. These conflicting results may be attributed to the small sample size and heterogeneity of enuretic patients.

Copeptin, which is cleaved from the C terminal portion of the AVP precursor protein, provasopressin, has recently been suggested as a useful biomarker of AVP levels because it exists in an equimolar ratio to AVP and is stable even after 1 week of storage at room temperature<sup>10</sup>. It has gradually replaced AVP measurement in the clinical setting, and several recent studies investigating the association between the AVP system and various clinical conditions have focused on copeptin rather than AVP<sup>11,12</sup>.

AQP2 is expressed in the cells of renal cortical and medullary collecting ducts and is regulated by AVP<sup>13</sup>. Recently, several investigators have examined both plasma copeptin and urinary AQP2 instead of plasma AVP in patients with nocturnal enuresis. Norbantoglu et al.<sup>14</sup> first reported that plasma copeptin was significantly lower in enuresis patients than controls. Valenti et al.<sup>15</sup> showed that the day/night (D/N) urinary AQP2 ratio was higher in enuretic patients with nocturnal polyuria, indicating lower relative levels of AQP2 at night, and that alterations of this ratio correlated with enuresis severity.

Here, we report a prospective investigation of copeptin and AQP2 levels in MNE patients (University Hospital Medical Information Network clinical trial registry, number UMIN000016915). Changes in circadian pattern of plasma copeptin and urinary AQP2 during the course of dDAVP treatment were investigated.

## **Material And Methods**

### *Subjects.*

This study protocol was approved by Juntendo University Ethical Committee, and the study was conducted at Juntendo University Nerima Hospital (Tokyo, Japan).

A detailed clinical history was obtained from patients and their parents, and a physical examination was performed at our outpatient clinic. Patients were instructed to ensure sufficient fluid intake during the morning and early afternoon, to empty the bladder and restrict fluid and fruit intake before going to bed at night, and to keep a 2-week bladder diary at home.

Included nocturnal enuresis patients were aged 6–11 years without history of previous enuresis treatment, with nocturnal polyuria (**defined by the Japanese criteria: the nocturnal volume of the patients exceeds 0.9 ml/kg/sleeping hour or 250 ml**)<sup>16</sup>. Patients with other lower urinary tract symptoms, **as determined using the checklist from Vande Walle et al.**<sup>1</sup> were excluded.

### *Study design.*

dDAVP orally disintegrating tablet (ODT; dose 120 µg) was administered 30–60 min before bedtime daily for 4 weeks, after which each child's response was assessed according to International

Children's Continenence Society standardization guidelines<sup>17</sup>: non-response (NR: 0%–49% decrease in wet nights/week), partial response (PR: 50%–99% decrease), and complete response (CR: 100% decrease).

In patients who did not achieve CR at 4 weeks, ODT dose was increased to 240 µg and results were evaluated after 4 weeks. Patients were then classified into three groups:

CR at 120 µg ODT (120R)

CR at 240 µg ODT (240R)

NR at 240 µg ODT (240NR)

In responders (120R/240R), we tapered ODT doses every 4 weeks to try to maintain CR: 240 µg ODT/day → 120 µg ODT/day → 60 µg ODT/day → 60 µg ODT/alternate day → cessation.

Urine and blood samples were obtained from all patients at home with assistance from their parents before and during the course of treatment (every 4 weeks after ODT doses had been changed). At each point, samples were taken once in the late afternoon when patients came home from school without prior oral intake >2 h (Day-sample), and once upon waking up in the morning (Night-sample). Urine samples (~10 ml) were collected, and blood samples were taken using the finger-prick procedure. Four drops of capillary blood (~100 µl), were put in a special test tube (Eiken Chemical Co Ltd, Tokyo, Japan) containing plasma-separating material. After centrifugation at 3000 g for 3 min, nearly 50 µl of plasma samples were obtained.

Test tubes containing urine and plasma were kept at 4°C and brought to the outpatient clinic for analysis within 24 h. The percentage of dry nights was calculated using diary data from the previous 4 weeks.

Urine examination

Part of each urine sample was processed for routine laboratory examinations including urinary osmolality, sodium, creatinine, calcium, and nitrogen levels; the remainder was stored at –80°C for AQP2 measurement.

*Urine processing*

To semi-quantify the amount of AQP2 excreted in the urine, the day and night urine samples from

each patient were spun down at 2000 g for 30 min at 4°C to remove cellular debris. One milliliter of supernatant was obtained and added to 1 ml of total exosome solution (Invitrogen No. 4484452) and incubated for 1 h at room temperature. This was spun down at 10000 g for 1 h at 4°C. After removing the supernatant, the remaining pellet was spun down at 10000 g for 5 min at 4°C. The supernatant was discarded. The pellet was added to 50 µl of sample buffer and incubated for 30 min at 37°C. This sample was subjected to immunoblot analysis.

#### *Immunoblotting*

The immunoblotting of AQP2 was done based on the methods of Valenti et al<sup>15</sup>. Primary antigen was the 1:1000 dilution of rabbit anti-human AQP2 (Millipore Lot.2556276), and secondary antigen was the 1:5000 dilution of anti-rabbit (SIGMA A0545). Antigen–antibody reactions were visualized using HRP solution. Ten microliters of processed urine samples were loaded on the gels and AQP2 was analyzed semi-quantitatively by Western blotting. Densitometric units obtained for the 29-kD band of AQP2 were standardized to urine creatinine concentration<sup>18</sup>. Finally, the ratio between the densitometric units of the daytime 29-kD band of AQP2 versus the nighttime 29-kD band was calculated for each patient. Density was measured by densitometry and quantified using NIH software (Image J 1.48).

#### *Plasma copeptin measurement*

Plasma copeptin was measured using a novel commercial chemiluminescence assay (Cloud Clone Corp, SEA 365Hu, USA). The analytical detection limit of the assay is 6.2 pg/ml (range 15.6–1000 pg/ml).

#### *Statistical methods*

Data was analyzed using Windows PC software JMP 13 (SAS Co Ltd, USA). Between-group differences were assessed using student t tests for parametric data and Mann–Whitney U-test and Wilcoxon signed-rank test for nonparametric data. Correlations between variables were evaluated using Pearson’s correlation coefficient. Statistical significance was defined as a p value of <0.05.

## **Results**

Thirty-two children (23 boys and nine girls; mean age 8.1 y) with MNE provided informed consent (from patients and parents) to participate in the study and were enrolled. Eight patients did not complete the study (two did not attend appointments; six failed to take blood and urine samples at home). Therefore, data analysis was carried out on 24 patients (16 boys and eight girls). There were no differences in baseline characteristics between subjects who completed the study and those who discontinued (Table 1).

Of the 24 subjects who completed the study, 15 were dDAVP responders (120R and 240R) and nine were non-responders (240NR). There were no differences between dDAVP responders and non-responders in terms of their age, number of wet nights, bladder capacity and maximal voided volume (Table 2). Retrospective analysis revealed that dDAVP responders required ODT  $6.64 \pm 2.39$   $\mu\text{g}/\text{kg}/\text{day}$  to achieve CR for enuresis; dDAVP non-responders remained enuretic with the maximal dose of  $9.08 \pm 3.06$   $\mu\text{g}/\text{kg}/\text{day}$  ODT ( $p=0.04$ ). **The mean nocturnal urine volume (ml/kg/sleep hour) of responders was decreased from  $1.16 \pm 0.24$  to  $0.56 \pm 0.05$  ( $p=0.0005$ , Wilcoxon signed-rank test), whereas that of non-responders was decreased from  $0.93 \pm 0.03$  to  $0.75 \pm 0.03$  ( $p=0.13$ , Wilcoxon signed-rank test).**

Five of the responders (33%) relapsed during dose tapering: one on 120  $\mu\text{g}$  ODT/day and four on 60  $\mu\text{g}$  ODT/alternate day treatment. The 10 remaining patients achieved complete resolution of enuresis with dDAVP treatment, which persisted following cessation of therapy.

#### *Urine Examination*

Before dDAVP treatment, there were no significant differences in terms of urine gravity, osmolality, and calcium/creatinine ratio between dDAVP responders and non-responders (Table 2). There were also no significant differences in D/N ratios of urinary AQP2 between responders and non-responders, indicating a lack of predictive power.

After 8 weeks' treatment with dDAVP, the D/N ratios of urinary AQP2 and percentage of dry nights were inversely correlated, i.e. lower relative nighttime AQP2 was associated with more wet nights after treatment (Fig 1;  $p=0.00000069$ ). Changes of D/N ratios of urinary AQP2 before and during

dDAVP treatment (when CR was achieved) for the 120/240R and 240NR groups are shown in Figure

2. There was a significant decrease of D/N ratios of urinary AQP2 in responders only.

In dDAVP responders without subsequent relapse, the D/N ratios of urinary AQP2 were further decreased and remained low even after the cessation of dDAVP treatment (Fig 3a); however, in responders with subsequent relapse, these ratios were not decreased under dDAVP treatment (Fig 3b).

#### *Blood Examination*

Baseline serum creatinine and electrolyte concentrations were not significantly different between responders and non-responders (Table 3).

Pre-treatment D/N ratios of plasma copeptin were significantly higher (i.e. relative nighttime copeptin levels were lower) in dDAVP non-responders (240NR) than in dDAVP responders (120R) (Fig 4).

**In addition, we found that the patients whose pretreatment D/N ratio of plasma copeptin less than 2.30 will expect good dDAVP response (sensitivity 57.14% and specificity 91.67%).**

Pre-treatment D/N ratios of plasma copeptin were also higher in the non-responder (240NR) group compared with the subgroup of responders who did not relapse after dDAVP cessation, although the difference was not significant ( $p=0.14$ ). In dDAVP responders without subsequent relapse, the D/N ratios of plasma copeptin were also decreased after cessation of dDAVP treatment, as had been observed in D/N ratios of urinary AQP2. In contrast, in dDAVP responders with subsequent relapse, the D/N ratios of plasma copeptin did not decrease (data not shown).

#### **Discussion**

Plasma copeptin predicted response to dDAVP treatment in this study. A lower D/N ratio (i.e. higher relative nighttime copeptin) predicted better treatment response. Data from this biomarker also suggests that impaired endogenous AVP secretion in enuretic patients improves after successful dDAVP treatment for enuresis.

Increased nighttime urine output has long been regarded as an important factor in nocturnal enuresis<sup>19</sup> and may be predominantly attributable to decreased secretion of AVP<sup>20,21</sup>; the AVP analogue dDAVP is therefore an evidence-based first-line therapy (grade Ia evidence).



Approximately 30% of patients achieve total dryness using dDAVP, with perhaps another 40% exhibiting a significant decrease in nighttime wetting<sup>19</sup>, although the relapse rate after discontinuation is high (60%–70%)<sup>22</sup>.

Many investigators have hypothesized that endogenous AVP secretion restores in patients who are successfully treated with dDAVP, but data on this issue have been controversial. Knudsen et al. reported that endogenous secretion of AVP in eight MNE patients did not change after 24 weeks of dDAVP therapy<sup>23</sup>, whereas Chiozza et al.<sup>24</sup> showed that endogenous secretion of AVP in 25 enuretic patients was decreased before treatment and restored after dDAVP therapy. In the present study, we examined plasma copeptin, a stable and sensitive surrogate marker for AVP release before and after dDAVP therapy in MNE patients. In 2013, a Turkish group investigated the circadian rhythm of plasma AVP and copeptin; morning (7am) plasma copeptin – but not AVP - was decreased in enuretic patients before treatment<sup>14</sup>. We have conducted a prospective clinical study of plasma copeptin measurements in our enuretic patients before, during, and after dDAVP treatment. The sampling was performed at home by the patients' parents at two points (night and day). The D/N ratio of the plasma copeptin before treatment was low in dDAVP responders but high in non-responders. The ratios were significantly decreased after treatment in dDAVP -responders. These results indicate that the D/N ratio of plasma copeptin before treatment predicts response to dDAVP therapy in enuretic patients. In addition, we found that endogenous AVP, assessed indirectly by copeptin level measurements, restores in patients who maintain dryness after cessation of dDAVP treatment. This in part explains the mechanism by which dDAVP improves enuresis in a large proportion of patients, even after treatment discontinuation.

In the present study, we also measured urinary AQP2, another marker reflecting AVP profiles. A previous study by Radetti et al.<sup>25</sup> showed that nocturnal urinary AQP2 was lower in enuretic patients, but statistical significance was only seen in dDAVP responders. However, Kamperis et al.<sup>26</sup> did not find any differences in nocturnal urinary AQP2 between enuretic patients and controls. Recently,

Valenti et al.<sup>15</sup> calculated D/N ratios of urinary AQP2 in their patients and found that these ratios were higher in enuretic patients.

In our study, we confirmed D/N ratios of urinary AQP2 were decreased after successful treatment with dDAVP. Both the D/N ratios of plasma copeptin and urinary AQP2 reflected enuresis severity. dDAVP administration does not influence circulating copeptin<sup>27</sup>, but it increases AQP2 in murine kidney collecting duct cells<sup>28</sup>. These findings indicate that plasma copeptin might be the more appropriate predictor of dDAVP treatment response for enuresis, especially during treatment, although the measurement of urinary AQP2 has some practical advantages over plasma copeptin due to its less invasive sampling process.

Although this study was conducted in a small number of patients, we found that the D/N ratios of plasma copeptin and urinary AQP2 are important therapeutic indices in enuresis treatment. **There are several limitations. The subject of the current study was patients with MNE only. We acknowledge the possibility of day-to-day variation. Since the samplings of blood from the fingertips of the patients were rather invasive, we only could analyze the data from the limited numbers of available samples. We need to extend this study with larger sample sizes in the future.**

## **Conclusions**

This study investigated novel biomarkers of dDAVP treatment for nocturnal enuresis. The D/N ratio of plasma copeptin before treatment may predict response to dDAVP treatment and D/N ratio of urinary AQP2 is useful in monitoring endogenous AVP secretion during dDAVP treatment.

## **Acknowledgments**

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## **List of Abbreviations**

dDAVP = desmopressin acetate

CR = complete response

MNE = monosymptomatic nocturnal enuresis

NR = non-response

ODT, orally disintegrating tablet

AVP = arginine vasopressin

AQP2 = aquaporin 2

PVDF = polyvinylidene fluoride

**MVV= maximal voiding volume**

**NUV= nocturnal urine volume**

### **Figure Legends**

Figure 1: The relationship between the D/N ratios of urinary AQP2 and % of wet nights in all subjects.

Pearson's correlation coefficient test,  $r = 0.5558$ ,  $p = 0.00000068$ .

Figure 2: The changes of D/N ratios of urinary AQP2 before and during dDAVP treatment in dDAVP responders and non-responders.

The bold lines indicate average value in each group. Wilcoxon signed-rank test,  $p = 0.0004$ .

Figure 3: The changes of D/N ratios of urinary AQP2, before and after dDAVP treatment in dDAVP responders without subsequent relapse (Figure 3a), and before dDAVP treatment and at relapse in dDAVP responders with subsequent relapse (Figure 3b).

The bold lines indicate average value in each group. Wilcoxon signed rank test,  $p = 0.005$

Figure 4: The D/N ratios of plasma copeptin before treatment in 120R, 240R and 240NR.

Kruskal–Wallis test, Mann–Whitney's U-test,  $p = 0.02$

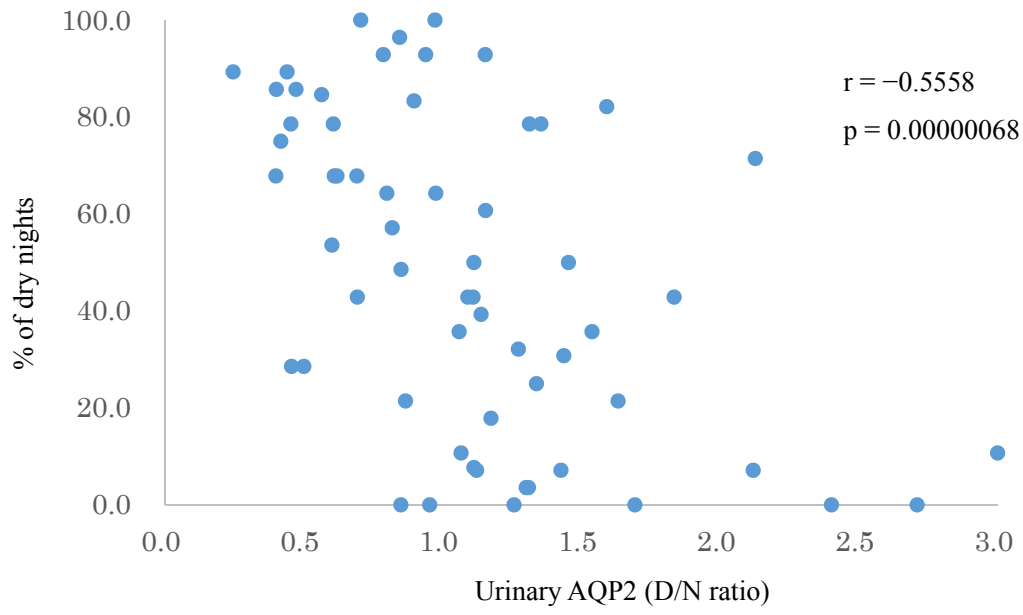
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Figure 1

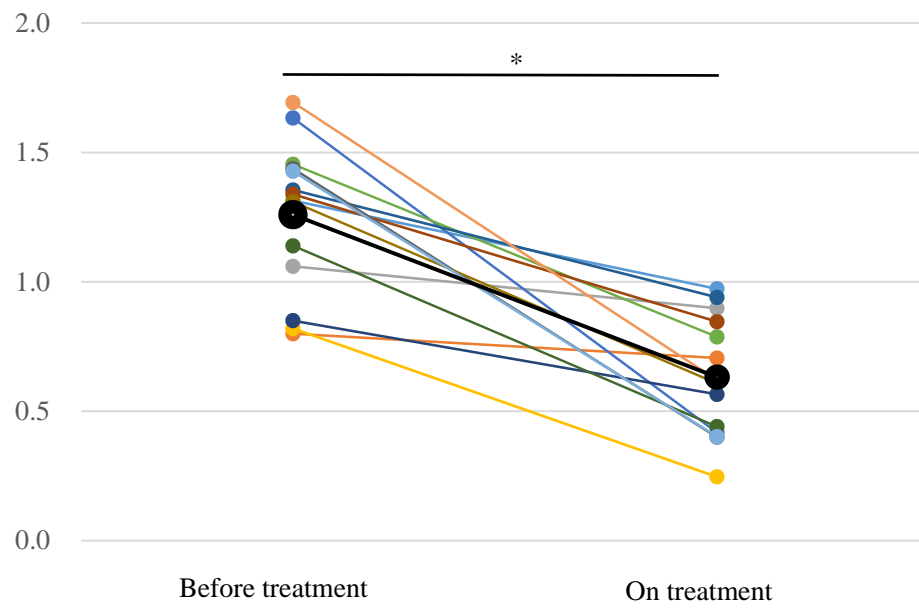




\*p = 0.0004

Figure 2

dDAVP responder



dDAVP non-responder

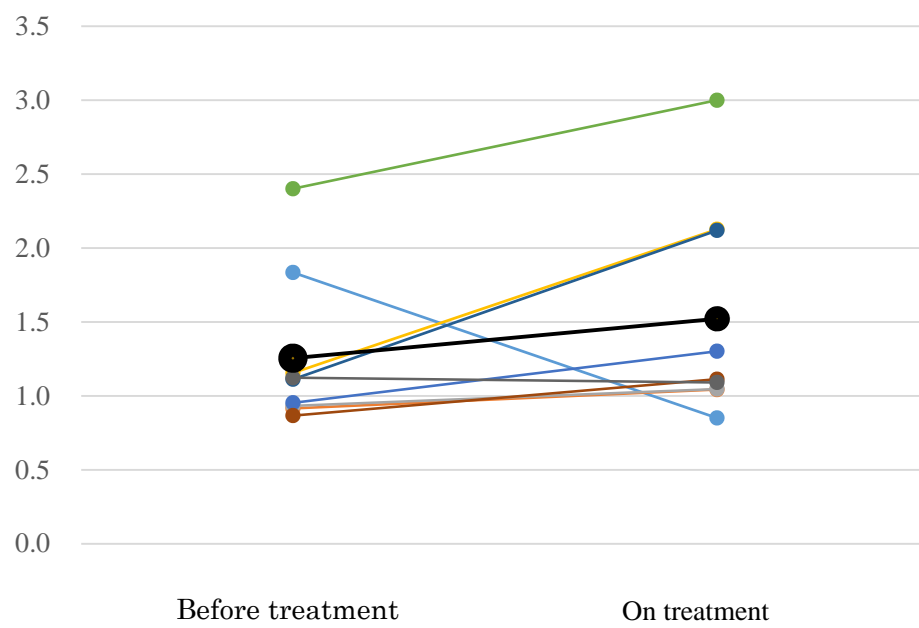


Figure 3-a

\*p = 0.005

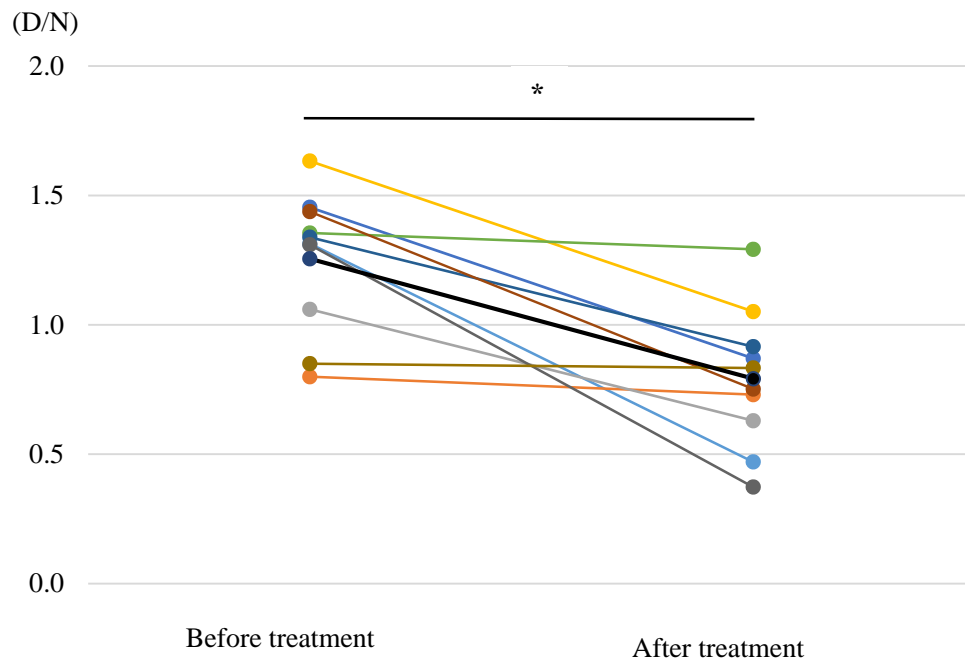


Figure 3-b

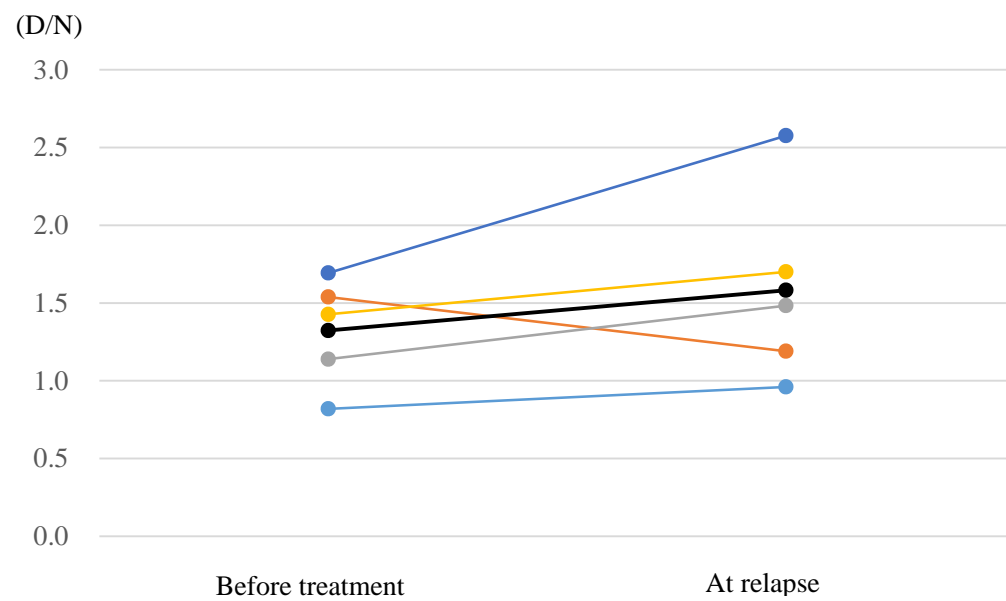
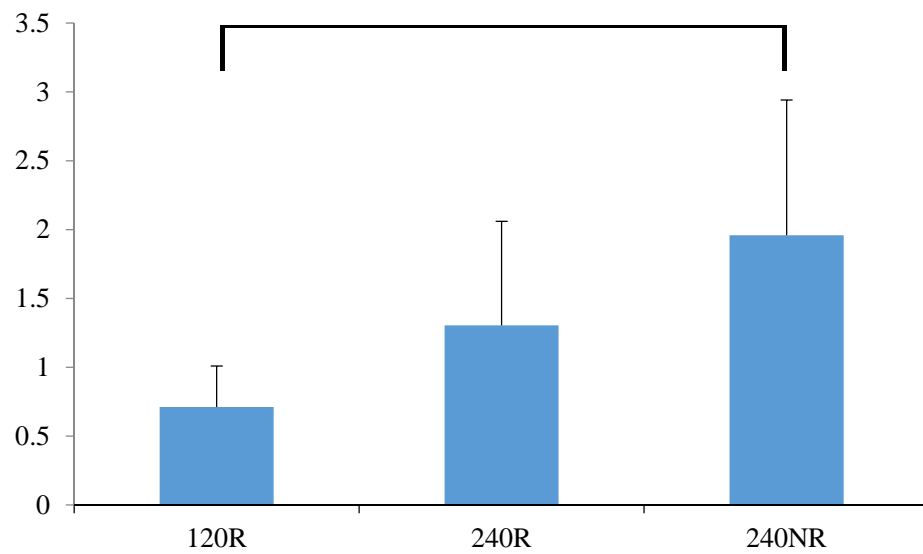


Figure 4

(D/N)

\*p = 0.02



Number	Study-completed subject (n = 24)	Study-uncompleted subject (n = 8)	p value
gender (boy: girl)	16:8	6:2	p = 0.10
Age(years)	8.6 ± 1.6	7.6 ± 0.5	p = 0.05
Number of wet nights/week	5.1 ± 2.0	5.9 ± 2.0	p = 0.31
BW(kg)	30.1 ± 11.8	23.3 ± 5.2	p = 0.11
Family history	10/24	3/8	p = 0.59
NUV(ml/kg/sleep hour)	1.09 ± 0.23	1.18 ± 0.18	p = 0.21
MVV (ml/kg)	7.8 ± 2.2	10.5 ± 4.3	p = 0.12

Table 1. Individual patient characteristics. Average ± SD

Number	DDAVP Responder (n = 15)	DDAVP Nonresponder (n = 9)	p value
gender (boy: girl)	8:7	8:1	p = 0.12
Age(years)	8.5 ± 1.2	8.7 ± 2.0	p = 0.64
Number of wet nights/week	4.7 ± 2.1	6.1 ± 1.7	p = 0.14
BW(kg)	29.9 ± 9.1	30.4 ± 15.4	p = 0.58
Family history	6/15	4/9	p = 0.63
<b>NUV(ml/kg/sleep hour)</b>	<b>1.16 ±0.24</b>	<b>0.93 ±0.03</b>	<b>p = 0.16</b>
MVV (ml/kg)	8.8 ± 2.4	6.9 ± 1.5	p = 0.07
ODT (mcg/kg/day)	6.64 ± 2.39	9.08 ± 3.06	p = 0.04

Table 2. Patient data before dDAVP treatment. Average ± SD

	dDAVP Responder	dDAVP Non-responder	p value
Urine gravity	1.023 ± 0.007	1.023 ± 0.009	p = 0.86
Urine Osmolality (mOsm/kg)	730.8 ± 170.7	705.8 ± 217.0	p = 0.48
Calcium/Creatinine	0.13 ± 0.10	0.16 ± 0.11	p = 0.68
Urine β2 micro-globulin (μg/L)	77.1 ± 47.1	51.2 ± 27.6	p = 0.17
Serum Cre (mg/dL)	0.41 ± 0.08	0.48 ± 0.11	p=0.10
β2 micro-globulin (mg/L)	1.50 ± 0.29	1.53 ± 0.29	p = 0.75
Serum Na (mmol/L)	139.3 ± 1.54	140.0 ± 1.25	p = 0.23
Serum K (mmol/L)	4.3 ± 0.40	4.3 ± 0.41	p = 0.90
Serum Ca (mg/dL)	9.5 ± 0.31	9.6 ± 0.31	p = 0.22

Table 3. Biochemical examination data. Average ± SD