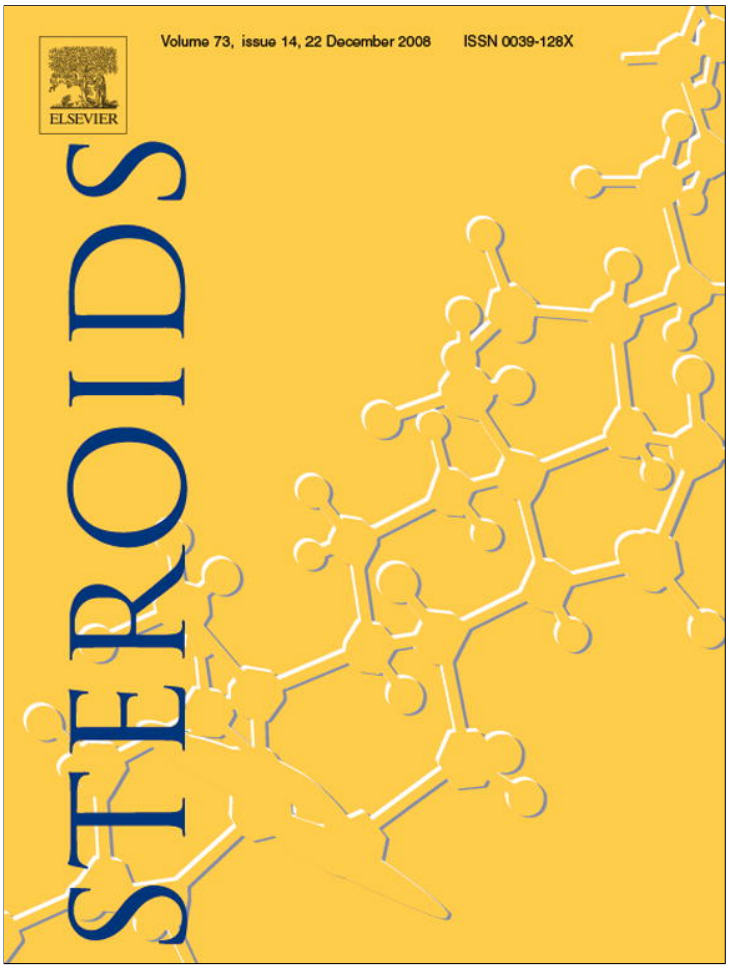

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Daily urinary free cortisol and cortisone excretion is associated with urine volume in healthy children☆

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ABSTRACT

Background: In experimental studies, a high fluid intake and a corresponding high urine volume have been shown to increase renal excretion rates of urinary free cortisol (UFF) and cortisone (UFE) in adults. We aimed to examine whether 24-h UFF and UFE excretion rates are also affected by urine volume in children.

Methods: In 24-h urine samples of 100 pre-pubertal and 100 pubertal healthy children UFF, UFE, tetrahydrocortisol (THF), 5α-tetrahydrocortisol (5α-THF), and tetrahydrocortisone (THE) were quantified by RIA. The sum of THF, 5α-THF, and THE, the 3 primarily glucuronidated tetrahydrometabolites (GC3), reflects daily cortisol secretion. Associations of urine volume with outcome variables UFF, UFE, and GC3 were examined in both developmental groups using multiple regression models adjusted for sex, body weight and height.

Results: Significant positive associations were observed between 24-h urine volume and UFF and UFE in both groups with the highest explained variation for UFE [partial $R^2 = 0.11$ in pre-pubertal group ($P < 0.005$); partial $R^2 = 0.15$ in pubertal group ($P < 0.0001$)]. However, for outcome GC3, urine volume was not significant in either of the groups.

Conclusion: Urinary 24-h excretion rates of UFF and UFE but not of the marker of glucocorticoid secretion are affected by daily urine volume in healthy free-living children. For a specific assessment of associations of UFF and UFE with (patho)physiologically relevant factors, urine volume should be considered as a confounder.

1. Introduction

Urinary measurement of glucocorticoid metabolites in 24 urine samples provide a time integrated, stress free, *in vivo* assessment of glucocorticoid status in healthy and ill children and adults. Additionally, it allows a simultaneous differentiation between the glucocorticoid secretion (adrenocortical activity) and the level of potentially biological active fraction of glucocorticoids [1]. Urinary free cortisol (UFF) is frequently used as an index for functional glucocorticoid activity in clinical laboratories. Recent findings suggested that urinary free cortisone (UFE) may be a useful complementary analyte to UFF

Abbreviations: BSA, body surface area; DONALD, Dortmund Nutritional and Anthropometric Longitudinally Designed; UFF, urinary free cortisol; UFE, urinary free cortisone; THE, tetrahydrocortisone; THF, tetrahydrocortisol; 5α-THF, 5α-tetrahydrocortisol; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2.

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for a more meaningful assessment of functional glucocorticoid activity [1–4]. In addition, the ratio of UFE and UFF has been used to assess renal 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) [5–7], which catalyses dehydrogenation of cortisol to cortisone. Nevertheless, high fluid intake has been reported to significantly increase UFF in healthy subjects [8–10]. Recently, in an experimental study in healthy men Fenske [8] found not only UFF, but also UFE to be dependent on urinary volume after water loading, with a prominent stimulation of UFE excretion. Accordingly it has been argued that the urine volume may constitute an important confounding factor when using UFF and UFE to assess glucocorticoid status [8]. Furthermore, if varying water loads affect UFF and UFE differently, the ratio of them as an index of 11β-HSD2 may also be confounded. Until now, no such examination has yet been undertaken regarding 24-h urine samples of free-living children. Thus we aimed to examine whether 24-h UFF, UFE, and glucocorticoid secretion marker are affected by urine volume in healthy children.

2. Subjects and methods

2.1. Study design and subjects

This cross-sectional study was carried out in 200 healthy children from the Dortmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Study. The DONALD study is an ongoing longitudinal (open cohort) study collecting detailed data on diet, growth, development, and metabolism between infancy and adulthood (once a year for subjects older than 2 years). Overall, the study population primarily comprises of middle class families with a relatively high socioeconomic status. In order to assess the state of health of the participants, a medical history is annually taken by the study pediatrician. This is completed by a physical examination and an assessment of pubertal development using the grading system defined by Tanner. Further details of the DONALD study are provided elsewhere [11,12]. The DONALD study is exclusively observational, non-invasive (until age 18) and approved by the Ethics Committee of the University of Bonn (Germany). All examinations and assessments are performed with parental, and later on with the children's written consent.

For the present evaluation, 200 children were randomly selected from the DONALD cohort in order to examine two commensurate groups: a pre-puberty and a puberty group. The inclusion criteria were as follows: The pre-pubertal and pubertal children had to have Tanner stage 1 and Tanner stage 3–5 for breast or penis development, respectively. The included age ranges were 8–9 years and 12–14 years, with girls in the puberty group being 12–13 years and boys being 13–14 years old. Each developmental group comprised of 50 boys and 50 girls.

2.2. Measurement and assessment

Body weight was measured to the nearest 0.1 kg with an electronic scale (Seca 753, Seca weighing and measuring systems, Hamburg, Germany). Height was measured in the standing position to the nearest 0.1 cm with a digital telescopic wall-mounted stadiometer (Harpenden, Holtain Ltd., Crosswell, UK). UFF, UFE, tetrahydrocortisone (THE), tetrahydrocortisol (THF), and 5α-THF were measured by specific radioimmunoassays with the use of tritiated steroids (Amersham Pharmacia Biotech, Freiburg, Germany) and specific antibodies raised and characterized in the steroid laboratory in the department of pharmacology (University of Heidelberg, Germany), as described elsewhere [13]. Before radioimmunoassay, UFF and UFE were extracted from the urine with dichloromethane and chromatographically purified using Celite columns (Celite columns 545 AW; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). THE, THF, and 5α-THF were quantified after treatment with glucuronidase (Roche Diagnostics GmbH, Mannheim, Germany) in a final dilution of 1:1200 (vol:vol). Intra- and interassay CVs were <10% and <13%, respectively. Creatinine was measured by the Jaffé method [14] with the use of a creatinine analyser (Beckman-2; Beckman Instruments Inc., Fullerton, CA).

To assess the adrenal gland's total daily glucocorticoid secretion, the 24-h excretion rates of the analysed major urinary glucocorticoid metabolites THE, THF, and 5α-THF, most of which are excreted as the glucuronide conjugates, were summed and referred to as GC3 [15,16]. This sum comprises ~50% of the overall amount of glucocorticoids secreted per day [15] and is therefore a reasonable estimate of total cortisol and cortisone secretion, but not necessarily an appropriate estimate of bioavailable or potentially bioactive glucocorticoids [1,3,11,17]. Potentially bioactive free glucocorticoids were assessed by the sum of UFF and UFE (UFF + UFE) [1,11]. The ratio of UFE and UFF (UFE/UFF) was used as an index for 11β-HSD2.

2.3. Statistical analysis

All statistical analyses were carried out with SAS procedures (Version 8,2, Statistical Analysis System). Data were presented as means ± S.D. $P < 0.05$ was considered statistically significant. Overall age group differences were tested using unpaired *t*-test. Preliminary covariance analyses (ANCOVA) were used to check for volume-by-sex und volume-by-group interactions. No volume-by-sex interaction was observed for any of the outcome variables, however volume-by-group interactions were observed for all of the outcome variables. Therefore the subsequent analyses were performed with boys and girls combined, but separately in pre-pubertal and pubertal group.

Association of urine volume with outcome variables UFF, UFE, UFF + UFE, UFE/UFF and GC3 were examined in multiple regression models adjusted for sex, body weight, and height. In regression models with UFF and/or UFE as an outcome, the inclusion of adrenal glucocorticoid secretion as one major influencing variable has been shown to allow identification of relevant determinants with less strong explanatory contributions [17]. In such models the glucocorticoid secretion level (GC3) may confound the associations of UFF and/or UFE with the potential determinants to be examined. Therefore, in a second run of the regression, GC3 was included as an additional independent variable in the models with outcome UFF, UFE, UFF + UFE and UFE/UFF. Independence of the variables were tested with multicollinearity test and no multicollinearity was observed. All variables used were also checked for normality and where required, log transformation was performed prior to the entering of the variables into

Table 1 – Demographic, anthropometic, and urinary characterization of 200 healthy children and adolescents according to age group

	Pre-puberty (8–9 years)	Puberty (12–14 years)	P[a]
n (male/female)	100 (50/50)	100 (50/50)	
Age (year)	8.5 ± 0.5	13.0 ± 0.7	<0.0001
Weight (kg)	29.6 ± 5.7	51.7 ± 9.6	<0.0001
Height (cm)	133.6 ± 6.4	162.7 ± 8.0	<0.0001
Total energy intake (MJ/d)	7.0 ± 1.5	8.5 ± 2.0	<0.0001
Urine volume (mL)	657.0 ± 226.5	932.8 ± 361.4	<0.0001
Creatinine (mmol/d)	4.9 ± 1.2	9.0 ± 2.5	0.0001
UFF (μg/d)	12.1 ± 6.0	15.8 ± 7.6	<0.0001
UFE (μg/d)	21.8 ± 8.9	33.2 ± 12.3	<0.0001
UFF + UFE (μg/d)	33.8 ± 13.5	49.1 ± 18.7	0.0025
UFE/UFF	1.96 ± 0.65	2.31 ± 0.94	<0.0001
THF (mg/d)	0.8 ± 0.3	1.5 ± 0.55	<0.0001
THE (mg/d)	2.0 ± 0.8	3.5 ± 1.3	<0.0001
5α-THF (mg/d)	1.1 ± 0.5	2.1 ± 1.0	<0.0001
GC3 (mg/d)	3.9 ± 1.3	7.1 ± 2.6	<0.0001

UFF, urinary free cortisol; UFE, urinary free cortisone; UFF/UFE, the ratio of urinary free cortisol and urinary free cortisone; THF, tetrahydrocortisol; THE, tetrahydrocortisone; GC3, the sum of the 3 major glucocorticoid metabolites, THE, THF, and 5α-THF.

[a] *P* values by unpaired *t*-test for the difference between the pre-pubertal and the pubertal groups

the models. Additionally, to avoid correlated measurement errors, which may occur if both the dependent and the major independent variables are renal excretion rates measured in the same urine samples, the daily excretion rates were not determined conventionally. Instead they were determined as follows: each individually calculated 24-h analyte/creatinine ratio was multiplied by individual body weight and by published constant sex- and age-specific body weight-related creatinine reference values [18] and this yields the corresponding creatinine-standardized 24-h analyte excretion rate [1,17].

3. Result

Demographic, anthropometric, dietary [19] and urinary data of 200 healthy subjects are shown in Table 1 according to age groups. Group means were significantly different between pre-pubertal and pubertal groups for all of the variables ($P < 0.005$).

Table 2 reports the results for the association of urine volume with free glucocorticoids in 24-h urine in a regression model adjusted for sex, weight, and height. Significant positive associations were observed between 24-h urine volume and UFF as well as UFE in both pre-pubertal and pubertal groups with a higher explained variation for UFE, especially in the pubertal group ($\beta = 0.51$, $R^2 = 0.23$). Urine volume was positively associated with UFE/UFF in the pubertal, but not significantly in the pre-pubertal group. For outcome GC3, urine volume was not significant in either of the groups.

After including GC3 (adrenal glucocorticoid secretion) as a potential predictor, in models with outcome UFF and/or UFE, urine volume was still a significant predictor but with

Table 2 – Step-wise multiple linear regressions for urinary free glucocorticoids with the predictor 24-h urine volume

Outcome variables	Predictors	Pre-puberty			Puberty		
		β	R^2	P	β	R^2	P
UFF	A priori adjusted variables[a]		0.07			0.11	
	Urine volume	0.26	0.04	<0.05	0.31	0.06	<0.05
	Model R^2		0.11	<0.05		0.17	<0.005
UFE	A priori adjusted variables[a]		0.14			0.06	
	Urine volume	0.39	0.11	<0.005	0.51	0.23	<0.0001
	Model R^2		0.25	<0.0001		0.29	<0.0001
UFF + UFE	A priori adjusted variables[a]		0.11			0.07	
	Urine volume	0.34	0.11	<0.005	0.46	0.19	<0.0001
	Model R^2		0.22	<0.0001		0.26	<0.0001
UFE/UFF	A priori adjusted variables[a]					0.11	
	Urine volume			n.s.	0.20	0.05	<0.05
	Model R^2			n.s.		0.16	<0.005

24-h urine volume, UFF (urinary free cortisol) and UFE (urinary free cortisone) were log-transformed; UFE/UFF, the ratio of UFE to UFF; n.s., not significant.

[a] Sex, body height, and weight, for which the sum of partial R^2 is shown.

Table 3 – Step-wise multiple linear regressions for urinary free corisol and/or cortisone with 24-h urine volume and the additional predictor GC3

Outcome variables	Predictors	Pre-pub			Pub		
		β	R^2	P	β	R^2	P
UFF	A priori adjusted variables[a]		0.04			0.11	
	GC3	0.29	0.05	<0.05	0.75	0.24	<0.0001
	Urine volume	0.26	0.04	<0.05	0.21	0.03	<0.05
	Model R^2		0.16	<0.05		0.38	<0.0001
UFE	A priori adjusted variables[a]		0.15			0.06	
	GC3	0.33	0.07	<0.005	0.74	0.38	<0.0001
	Urine volume	0.39	0.11	<0.005	0.41	0.15	<0.0001
	Model R^2		0.33	<0.0001		0.59	<0.0001
UFF + UFE	A priori adjusted variables[a]		0.12			0.06	
	GC3	0.31	0.07	<0.005	0.74	0.38	<0.0001
	Urine volume	0.34	0.11	<0.005	0.36	0.12	<0.0001
	Model R^2		0.30	<0.0001		0.56	<0.0001
UFE/UFF	A priori adjusted variables[a]					0.11	
	GC3			n.s.		n.s.	
	Urine volume			n.s.	0.20	0.05	<0.05
	Model R^2			n.s.		0.16	<0.005

24-h urine volume, GC3, UFF (urinary free cortisol) and UFE (urinary free cortisone) were log-transformed; UFE/UFF, the ratio of UFE to UFF; n.s., not significant.

[a] Sex, body height, and weight, for which the sum of partial R^2 is shown.

a decreased partial R^2 (UFE: $\beta = 0.41$, $R^2 = 0.15$). At the same time GC3 itself became a highly significant predictor for UFF ($\beta = 0.75$, $R^2 = 0.24$) and UFE ($\beta = 0.74$, $R^2 = 0.38$) in the pubertal group. In the pre-pubertal group GC3 also significantly contributed to UFF and UFE, however, the explained variation was less and the partial R^2 of urine volume remained unchanged (Table 3). Inclusion of GC3 did not alter the relationship between urine volume and UFE/UFF. In Fig. 1 associations of 24-h urine volume with the logarithm of urinary excretion rates of UFF and UFE from simple regression analysis are shown for the total sample ($n = 200$). To determine the relative changes of UFF and UFE per 100 mL urine volume, we performed a multiple regression analyses for the total sample adjusted for age, sex, and weight. The results derived from the corresponding regression equation are daily UFF increases by 3% and UFE by 5% with each 100 mL increase in urine volume.

4. Discussion

Our results indicate that in healthy children both daily urinary free cortisol and free cortisone excretion are positively correlated with urine volume, with a prominent association of volume and UFE. The sum of the 3 major urinary glucocorticoid metabolites THE, THF, and 5α-THF (GC3), which reflect adrenal gland's total daily glucocorticoid secretion, are not related to urine volume. These findings confirm that in free-living healthy children urine volume is not only a confounder of UFF in overnight samples [10] but also of UFF and/or UFE in 24-h urine samples. Additionally, urine volume or fluid intake does not seem to affect adrenal cortisol secretion in children. This is in agreement with the result of a recent experimental study in healthy adults [9].

In the regression model without considering glucocorticoid secretion, we found a much higher contribution of urine volume to UFF and UFE in the pubertal group than in the pre-pubertal group. After taking the influence of glucocorticoid secretion into account in the second run, this difference between the two development groups existed no longer. We also observed a much greater effect of adrenal glucocorticoid secretion (GC3) on UFF and/or UFE in the puberty group than in the pre-pubertal group. This may be due to the fact that the influence of adrenal glucocorticoid secretion and peripheral glucocorticoid metabolism on UFF and/or UFE is changing during growth despite a constant body surface area (BSA)-corrected excretion of UFF and/or UFE [3]. BSA-corrected glucocorticoid secretion is significantly higher in pubertal than in pre-pubertal children [20]. Therefore, an adjustment for glucocorticoid secretion is necessary in order to identify specific effects of influencing factors (other than glucocorticoid secretion itself) on free glucocorticoids in pubertal children. This finding also confirms that the adrenal glucocorticoid secretion, as one of the most important determinants of UFF and/or UFE, constitutes a confounder for the study of associations between nutritional factors and functionally free glucocorticoids [17].

Although several studies in adults report an influence of fluid intake and the corresponding urine volume on UFF, a recent study in 88 healthy women did not find this significant effect. Whether this might be due to additional metabolic influence on cortisol metabolism such as an increase in 11β-HSD2 activity (increase in renal cortisol to cortisone conversion) in women with elevated body fat [21] is unknown at present. In fact, the contribution of water load or urine volume to UFE is stronger than to UFF. This finding is in agreement with the result of an experimental study [9]. The author has suggested that water diuresis stimulates UFF and UFE by different mechanisms [8]. Water diuresis stimulates UFF mainly due to the escape of cortisol from reabsorption [9] in the

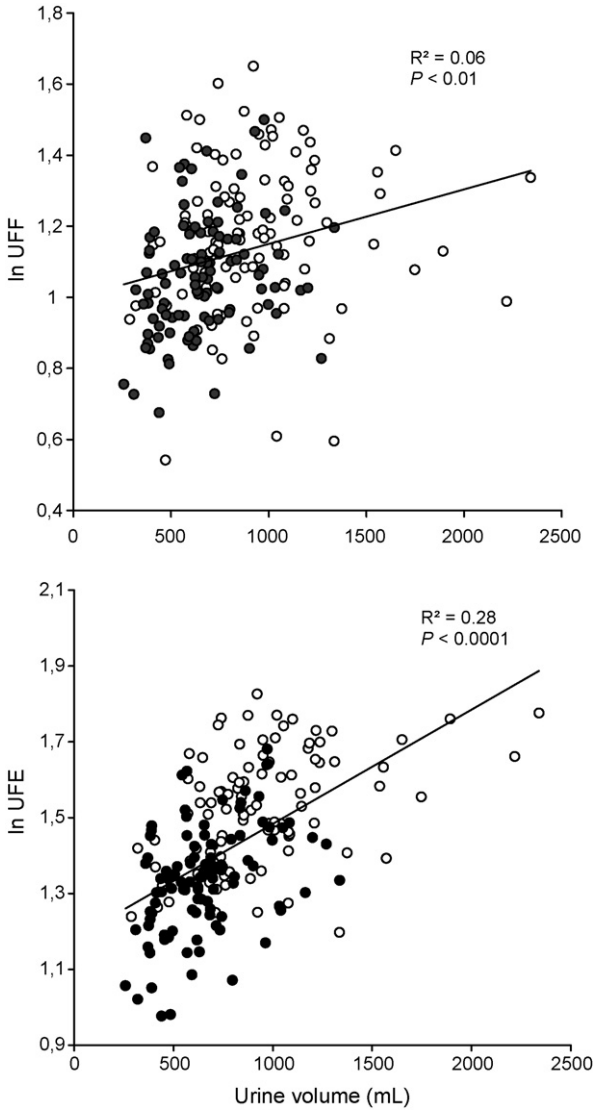

Fig. 1 – Association of 24-h urine volume with urinary excretion rate of UFF and UFE in 100 pre-pubertal (●) and 100 pubertal children (○). UFF, urinary free cortisol; UFE, urinary free cortisone. R^2 denotes the regression coefficient from simple regression analyses. Multiple regression analysis for all children adjusted for age, sex, and body weight yielded the following partial R^2: 0.03 for UFF ($P < 0.05$); 0.11 for UFE ($P < 0.0001$). Further results derived from the corresponding regression equation showed daily UFF increases by 3% and daily UFE increases by 5% with each 100 mL increase in urine volume.

proximal tubule [8]. Accordingly in a micropuncture experiment in rat kidney fractional cortisol reabsorption showed a comparable dependency on flow rate [22], which is determined by urine volume in the same period of urine collection, and the most predominant influence of flow rate on cortisol reabsorption was found in the proximal tubule in the same experiment [22]. Additionally, Mericq and Cutler assumed that the increase in urine cortisol excretion during high fluid intake may be caused by decreased renal metabolism of cortisol [9]. In contrast, Fenske [8] suggested an increased cortisol to cortisone metabolism by the 11β-HSD2, which leads to an augmented UFE excretion in the distal tubule. Cortisone production and consequently UFE excretion mainly occurs in the distal part of the tubule, however, also the proximal tubule may be capable of converting cortisol to cortisone [23]. Until now there is no evidence for a specific transport system for cortisol and/or cortisone. The reabsorption of cortisol and excretion of cortisone may most probably base on solute–solvent coupling effect [22]. With increased water load, the reabsorption of water in the distal tubule decreases (in absolute terms) provided a higher portion of the elevated water load has already been proximally reabsorbed. Thus, the cortisone enriched in the 11β-HSD2 containing cells of the distal tubule could be easier excreted against less fluid influx. Hence we prefer to the hypothesis that alteration of both UFE and UFF excretion with urine volume depends on transcellular water flow in the renal tubules. Since our results suggest differently strong effects of urine volume on UFF and UFE, UFE/UFF as an index for 11β-HSD2 can be confounded by variation in water load.

In contrast to low clearance values for cortisol and its non-conjugated metabolites, conjugated metabolites are cleared at rates approaching glomerular filtration [24–27]. This means there is only a minimal reabsorption of conjugated metabolites and thus their urinary excretion should not depend on water flow in the tubules. Our observation along with the data of Mericq and Cutler [9] are consistent with such a mechanism explanation. We found the indicator of glucocorticoid secretion (GC3), the sum 3 major urinary glucocorticoid metabolites THE, THF, and 5α-THF, which are primarily excreted as glucuronides in the kidney, is not related to urine volume.

Limitation of our study is its cross-sectional design. Although we have taken the effect of the varying glucocorticoid secretion rates between individuals on their UFF and UFE excretions into account, other possible relevant factors such as individual social or other stress forms or specific glucocorticoid metabolic enzyme activities could not be directly measured. However, it is certainly advantageous that our free-living children were not under experimentally induced stress, e.g., caused by manipulations of water intake. It should also be mentioned that the elaborate design of the DONALD study results in a selected population with a high educational attainment and socioeconomic status. Thus, our study does not include a large percentage of children with particularly unfavourable dietary habits, e.g., an extremely low fluid intake. However, this non-representativeness is less relevant for the present physiological examination and will likely result in underestimation rather than overestimation of the "true" associations.

Because of the limited age range of our subjects (8–9 and 12–14 years) and the cross-sectional study design, we cannot give clinical advice in detail. But we have been able to show that in healthy children with each 100 mL increase of urine volume, daily UFF increases approximately by 3% and UFE by 5%. This means that a child with high urine volume of 2 L, instead of a fictitious average volume of 1 L, would increase its daily UFF by approximately 30%. This might be of relevance for clinical diagnosis.

In conclusion, in healthy children, water load appear to affect both urinary free cortisol and cortisone, with a stronger influence on cortisone. In future studies and clinic diagnostics

using UFF and/or UFE, urine volume should be considered as a confounder. Adrenal gland's total daily glucocorticoid secretion, reflected by the sum of major urinary glucocorticoid metabolites (excreted in conjugated form in the urine) was not related to urine volume. Further studies are needed to investigate whether urine excretion of other steroid hormone metabolites may also be confounded by water load.

Disclosure

The authors have nothing to disclose.

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