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ORIGINAL ARTICLE

Significantly high level of late-night free cortisol to creatinine ratio in urine specimen in patients with subclinical Cushing's syndrome

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Summary

Objective Absence of a late-night cortisol nadir is a consistent biochemical abnormality in patients with cortisol-producing adenoma. We evaluated the abnormality of late-night urinary free cortisol to creatinine ratio (late-night UFCCR) in patients with subclinical Cushing's syndrome (SCS).

Methods Fifty-eight patients with incidentally detected adrenocortical adenomas [SCS: 9; nonfunctioning adenoma (NF): 49] were enrolled as subjects. Values measured in all patients were urinary free cortisol accumulated between 9:00 p.m. and 11:00 p.m. (late-night UFCCR), serum cortisol at 11:00 p.m. (midnight serum cortisol: MSC), serum cortisol after 1-mg overnight dexamethasone suppression test (1 mg-DST) and 24-h urinary free cortisol (UFC).

Results Median late-night UFCCR value in SCS was significantly higher than that in NF ($P < 0{\cdot}001$). Significant correlations were observed between late-night UFCCR and each of serum cortisol after 1 mg-DST and MSC ($r = 0{\cdot}537$, $P < 0{\cdot}001$ and $r = 0{\cdot}556$, $P < 0{\cdot}001$, respectively). There was no significant correlation between serum cortisol after 1 mg-DST and 24-h UFC ($r = 0{\cdot}211$, $P = 0{\cdot}112$). In receiver operating characteristic analysis for diagnosis of SCS, the areas under the curves of late-night UFCCR and 24-h UFC were 0·937 (95% confidence interval 0·865–1·008) and 0·726 (0·874–0·999), respectively. Late-night UFCCR cut-off value of 4·9 nmol/μmol Cre showed a sensitivity of 100% and a specificity of 76·6%.

Conclusion Patients with SCS showed higher late-night UFCCR values than those with NF. Late-night UFCCR was significantly correlated with autonomous cortisol production findings. Diagnostic performance of late-night UFCCR was superior to 24-h UFC. These results suggest that late-night UFCCR might represent one of the simple and reliable tests for SCS diagnosis.

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Introduction

Subclinical Cushing's syndrome (SCS) is defined as an adrenal adenoma that exhibit autonomous glucocorticoid production without specific signs or symptoms of Cushing's syndrome with overt hypercortisolism.[1] SCS is diagnosed in 5% to 20% of patients with adrenal incidentaloma.[1–3] SCS leads to metabolic disorders such as hypertension, dyslipidaemia and/or glucose intolerance,[3–5] which in turn cause elevated incidence of cardiovascular morbidity and mortality in SCS patients.[4] Although we should aggressively diagnose that entity, diagnostic strategy to be used to identify SCS remains controversial.[6]

To ascertain the presence of subtle cortisol production, the same endocrine tests used for diagnosis of overt hypercortisolism are employed.[7–10] At present, the following criteria are used to make a diagnosis of subtle cortisol production:[7–10] reduced cortisol suppression after 1-mg overnight dexamethasone suppression test (1 mg-DST), low morning ACTH levels, high 24-h urinary free cortisol (UFC) and high midnight serum cortisol levels (MSC). Generally, the 1 mg-DST is understood to be the most effective method for investigation of autonomous cortisol secretion.[6,11,12] MSC is also a reliable test, because the loss of circadian rhythm with absence of a late-night cortisol nadir is a consistent biochemical abnormality in patients with cortisol-producing adenoma.[13,14] However, for measurement of MSC, patients must be hospitalized or visit the hospital repeatedly to have their blood samples drawn. The procedure thus has inherent limitations and can cause undue stress.

Measurement of urinary cortisol levels can be used to detect biologically active free fraction cortisol.[15] Twenty-four-hour UFC determination is a standard test for the diagnosis of cortisol

overproduction.[8,9] However, a complete collection of 24-h urine is inaccurate and potentially unreliable. Midnight urine cortisol to creatinine ratio is considered to be the simplicity of the collection and a useful method for assessment of overt Cushing's syndrome[16] and hypopituitarism.[16,17] Expectations are therefore that 2-h late-night urinary free cortisol to creatinine ratio (late-night UFCCR) could prove to be simple and effective in assessing loss of late-night serum cortisol nadir and useful for SCS diagnosis. Another advantage of utilization of this method is that patients can collect urine samples on their own without requiring hospitalization or stress of pain. Measurement of late-night UFCCR is considered to have several benefits including increased accuracy of diagnosis, improved quality of life for patients and positive effects on the medical economy. However, effectiveness of late-night UFCCR for SCS diagnosis had not been studied in detail to date. The aim of this study was to evaluate the abnormality of late-night UFCCR accumulated for 2-h between 9:00 p.m. and 11:00 p.m. in patients with SCS and investigate whether late-night UFCCR could have a role in the diagnosis of SCS.

Subjects and methods

We prospectively evaluated 100 patients with incidentally detected adrenal masses who were referred to our department at Hiroshima University Hospital during the period from April 2009 to April 2012. Excluded from the study were patients diagnosed as having aldosterone producing adenoma ($n = 19$), pheochromocytoma ($n = 10$), overt Cushing's syndrome ($n = 4$), cyst ($n = 3$), adrenal cortical carcinoma ($n = 2$), ganglioneuroma ($n = 1$), asymptomatic metastasis of other tumours ($n = 1$) and 24-h creatinine clearance (Ccr) below 20 ml/min ($n = 2$). Only patients with adrenocortical adenoma were selected for this study. When not pathologically proven, the diagnosis of adrenocortical adenoma rested on the following criteria: less than 10 Hounsfield units on unenhanced CT,[18] absolute percentage enhancement washout of greater than 60% at 10-min[18] or loss of signal intensity on opposed phase against in-phase chemical shift magnetic resonance imaging.[19] Ultimately, a total of 58 patients (mean age 62·1 ± 11·7 years; 32 male and 26 female) were enrolled in this study. All patients studied had never used any glucocorticoids that could affect the HPA axis. Written informed consent was obtained from all the patients at the time of clinical investigation, and the study was approved by the Hiroshima University Ethics Committee.

Clinical measurement, endocrinological measurement and assessment

All patients were hospitalized and assessed by clinical measurements and endocrine tests. Physical measurements were carried out, and blood samples collected after an overnight fast. Height, body weight and waist circumference of the subjects were measured using standard methods, and body mass index (BMI) was then calculated. HbA1c [Japan Diabetes Society (JDS), %] was measured by means of high-performance liquid chromatography using an automated analyzer. HbA1c (%) was estimated as National Glycohaemoglobin Standardization Program (NGSP) equivalent value (%), calculated on the basis of the formula HbA1c (%) = 1·02 × HbA1c (JDS,%) + 0·25%.[20] The 24-h UFC was determined on the basis of urine samples collected between 2:00 p.m. and the following day at 2:00 p.m. The normal value of 24-h UFC established in our laboratory is 31–222 nmol/day. To investigate circadian rhythms of serum cortisol and plasma ACTH levels, these components were examined at 7:00 a.m. and 11:00 p.m. Determinations of serum cortisol and plasma ACTH levels at 7:00 a.m. were performed at least 30 min after the subject rested in a supine position. After urine elimination at 9:00 p.m., urinary samples were collected at 11:00 p.m. for the measurement of late-night UFCCR. Late-night UFCCR was examined the day after 24-h urine collection. After 1-mg dexamethasone was administered orally at 11:00 p.m., blood samples were collected the following morning at 7:00 a.m. to determine serum cortisol concentrations (1 mg-DST). UFC levels were measured by radioimmunoassay method (Immunotec Inc., Quebec, Canada). The lower detection limit of UFC was estimated to be 13.8 nM. Serum cortisol was measured by ECLusys 2010 cortisol assay (Roche Diagnostics Co., Mannheim, Germany) and plasma ACTH was measured using immunoradiometric assay by ACTH IRMA MITSUBISHI (Mitsubishi Chemical Medience Co., Tokyo, Japan).

Subclinical Cushing's syndrome was diagnosed using criteria previously reported.[7] Briefly, the criteria include the following essential conditions: (i) presence of adrenal incidentaloma; (ii) lack of specific clinical findings of Cushing's syndrome; (iii) normal range of morning cortisol level; and (iv) serum cortisol level greater than 83 nM after 1 mg-DST. The Endocrine Society's guidelines refer to a cut-off value of 50 nM after 1 mg-DST.[8] We used, however, a cut-off value of 83 nM after 1 mg-DST based on the Japan Endocrine Society's criteria, because all patients enrolled in the study were Japanese.[7] SCS diagnosis was made based on the presence of all of criteria described above and at least one of the following biochemical parameters: (i) plasma ACTH level at 7:00 a.m. less than 2·2 pM; and (ii) serum cortisol level at 11:00 p.m. (MSC) more than 138 nM. The patients without SCS were diagnosed as having nonfunctioning adenoma (NF).

Statistical analysis

Results were expressed as mean values with standard deviation or median values with 95% confidence interval (CI). First, differences between the patients with NF and those with SCS were determined by Student's *t*-test or Mann–Whitney *U*-test. Categorized data were analysed by Fisher's exact test. Second, correlations between variables were examined by Spearman's correlation as appropriate. Finally, receiver operating characteristic (ROC) analysis was performed to compare diagnostic utility for SCS of late-night UFCCR with that of 24-h UFC. For all analyses, SPSS 19.0J for Windows (SPSS Inc., Chicago, IL, USA) was used, and P values $<0·05$ were considered significantly.

Results

Clinical characteristics of patients with SCS and NF

Forty-nine patients were diagnosed as having NF, and nine patients were diagnosed as having SCS. No significant differences were observed in age, gender, waist circumference, BMI, blood pressure, lipid levels, glucose levels or tumour size between the NF group and the SCS group, as indicated in Table 1. Numbers of those using medication did not significantly differ between the two groups.

Endocrinological results of patients with SCS and NF

Endocrinological results of the two groups are presented in Table 2. No significant difference was observed in serum cortisol at 7:00 a.m. between the two groups. Median values of MSC, serum cortisol after 1 mg-DST and 24-h UFC in the SCS group were significantly higher than those in the NF group. Median value of ACTH at 7:00 a.m. in the SCS group was significantly lower than that in the NF group. Remarkably, median values of late-night UFCCR were 3·7 (95% CI 2·7–5·0) nmol/μmol Cre in the NF group and 12·6 (6·6–15·4) nmol/μmol Cre in the SCS group, with significant differences observed between the two groups ($P < 0{\cdot}001$).

Table 1. Clinical characteristics of patients with SCS and NF

	NF	SCS	P value
Number (male/female)	49 (26/23)	9 (6/3)	0·352
Age (years)	62·5 ± 12·2	59·9 ± 8·7	0·459
BMI (kg/m^2)	24·6 ± 3·8	24·4 ± 2·4	0·842
Waist circumference (cm)	85·9 ± 11·7	86·5 ± 7·0	0·854
Systolic blood pressure (mmHg)	124 ± 19	127 ± 6	0·654
Diastolic blood pressure (mmHg)	73 ± 15	81 ± 9	0·062
Total cholesterol (mM)	4·70 ± 0·78	4·72 ± 0·59	0·943
HDL-cholesterol (mM)	1·41 ± 0·31	1·55 ± 0·41	0·389
Triglyceride (mM)	0·97 (0·76–1·33)	1·22 (0·80–1·89)	0·173
Fasting plasma glucose (mM)	5·7 ± 1·9	5·8 ± 1·1	0·959
HbA1c (%)	6·3 ± 1·1	6·7 ± 1·7	0·525
24-h Ccr (ml/min)	95·5 ± 26·6	90·1 ± 25·8	0·572
Tumor size (mm)	17·5 (14·4–25·1)	19·1 (14·4–24·5)	0·810
Antihypertensive drug, *n* (%)	23 (46·9)	6 (66·7)	0·201
Antihyperlipidemic drug, *n* (%)	19 (38·8)	1 (11·1)	0·107
Hypoglycemic drug, *n* (%)	12 (24·5)	2 (22·2)	0·627

NF, non-functioning adenoma; SCS, subclinical Cushing's syndrome; BMI, body mass index; Ccr, creatinine clearance.
Data are expressed as mean values ± SD or median values (interquartile). *P* values were determined by Student's *t* test or Mann-Whitney *U* test. Categorized data were analyzed by Fisher's exact test.

Table 2. Endocrinological results of patients with SCS and NF

	NF	SCS	P value
Serum cortisol at 7:00 a.m. (nM)	362 ± 113	323 ± 72	0·333
MSC (nM)	69 (51–91)	188 (142–236)	<0·001
Serum cortisol after 1 mg-DST (nM)	28 (18–39)	127 (101–229)	<0·001
ACTH at 7:00 a.m. (pM)	5·5 (3·8–6·8)	2·8 (1·7–4·1)	0·001
24-h UFC (nmol/day)	77·8 (60·5–110·3)	104·1 (82·1–199·3)	0·033
Late-night UFCCR (nmol/μmol Cre)	3·7 (2·7–5·0)	12·6 (6·6–15·4)	<0·001

NF, non-functioning adenoma; SCS, subclinical Cushing's syndrome; MSC, midnight serum cortisol; DST, dexamethasone suppression test; UFC, urinary free cortisol; UFCCR, urinary free cortisol to creatinine ratio.
Data are expressed as mean values ± SD or median values (interquartile). *P* values were determined by Student's *t* test or Mann-Whitney *U* test.

Correlations between late-night UFCCR and each of serum cortisol after 1 mg-DST and MSC

1 mg-DST is the most effective method for investigation of autonomous cortisol secretion. We thus calculated Spearman's correlations between late-night UFCCR and serum cortisol after 1 mg-DST. Late-night UFCCR was significantly correlated with serum cortisol after 1 mg-DST ($r = 0{\cdot}537$, $P < 0{\cdot}001$) (Fig. 1a). On the other hand, serum cortisol after 1 mg-DST was significantly correlated with each of MSC and ACTH at 7:00 a.m. ($r = 0{\cdot}688$, $P < 0{\cdot}001$ and $r = -0{\cdot}449$, $P < 0{\cdot}001$, respectively) (Fig. 1b,c). However, there was no significant correlation between serum cortisol after 1 mg-DST and 24-h UFC ($r = 0{\cdot}211$, $P = 0{\cdot}112$) (Fig. 1d). In addition, we calculated Spearman's correlation between late-night UFCCR and MSC. Late-night UFCCR was also significantly correlated with MSC ($r = 0{\cdot}556$, $P < 0{\cdot}001$) (Fig. 2).

Diagnostic performance of late-night UFCCR measurement for the diagnosis of SCS

To compare diagnostic performance between late-night UFCCR and 24-h UFC, ROC analysis was performed. For the diagnosis of SCS, the areas under the ROC curves of late-night UFCCR and 24-h UFC were 0·937 (0·865–1·008) and 0·726 (0·874–0·999), respectively (Fig. 3). Late-night UFCCR cut-off value of 4·9 nmol/μmol Cre showed a sensitivity of 100%, a specificity of 76·6% and a diagnostic accuracy of 79·3%. Late-night UFC cut-off value with the best combined sensitivity and specificity was achieved at the level of 7·4 nmol/μmol Cre with a sensitivity of 77·8%, a specificity of 95·9% and a diagnostic accuracy of 93·1%. On the other hand, 24-h UFC cut-off value of 48·6 nmol/day showed a sensitivity of 100%, a specificity of 16·0% and a diagnostic accuracy of 29·3%. Twenty-four-hour UFC cut-off value with the best combined sensitivity and

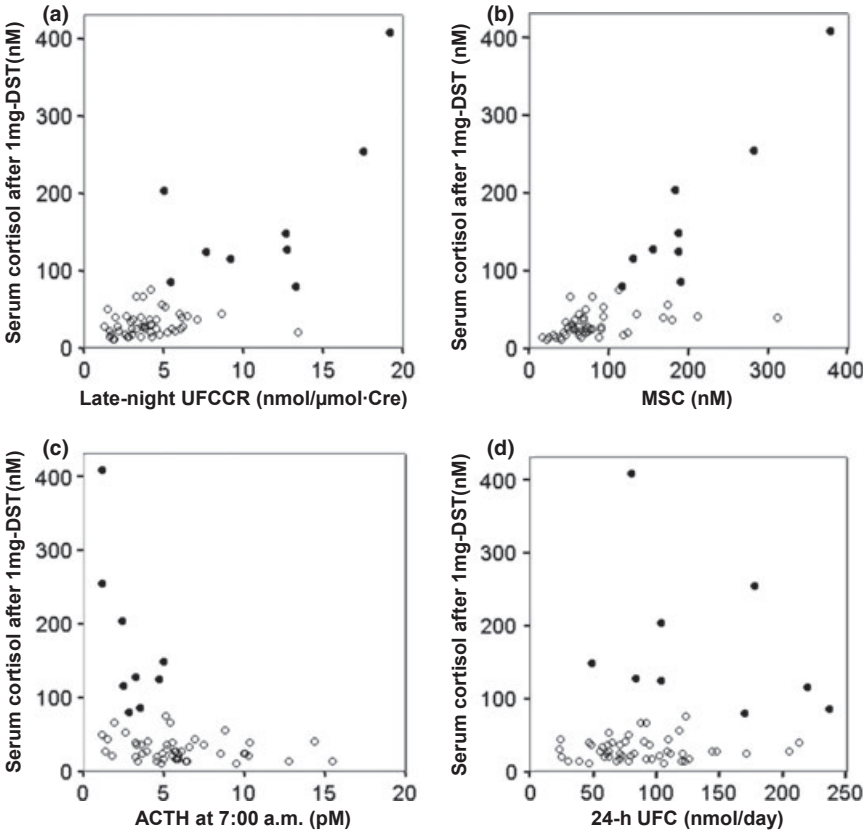

Fig. 1 Correlations among endocrinological parameters. (a) Correlation between late-night urinary free cortisol to creatinine ratio (UFCCR) and serum cortisol levels after 1-mg dexamethasone suppression test (1 mg-DST). Spearman's correlation coefficient between late-night UFCCR and serum cortisol levels after 1 mg-DST was 0·537, indicating significant correlation ($P < 0·001$). (b) Correlation between serum cortisol levels after 1 mg-DST and midnight serum cortisol (MSC) levels. Spearman's correlation coefficient between serum cortisol levels after 1 mg-DST and MSC levels was 0·688, indicating significant correlation ($P < 0·001$). (c) Correlation between serum cortisol levels after 1 mg-DST and ACTH levels at 7:00 a.m. Spearman's correlation coefficient between serum cortisol levels after 1 mg-DST and ACTH levels at 7:00 a.m. was −0·449, indicating significant correlation ($P < 0·001$). (d) Correlation between serum cortisol levels after 1 mg-DST and 24-h urinary free cortisol (UFC). Spearman's correlation coefficient between serum cortisol levels after 1 mg-DST and 24-h UFC was 0·211, which showed no significant correlation ($P = 0·112$). Open circles: nonfunctioning adenoma ($n = 49$); closed circles: subclinical Cushing's syndrome ($n = 9$).

specificity was achieved at the level of 102·9 nmol/day with a sensitivity of 66·7%, a specificity of 69·4% and a diagnostic accuracy of 69·0%.

Discussion

In this study, we demonstrated that patients with SCS showed higher late-night UFCCR values than those with NF, and late-night UFCCR was correlated with each of serum cortisol levels after 1 mg-DST and MSC levels, suggesting that late-night UFCCR expresses autonomous cortisol production. Accordingly, for the diagnosis of SCS, diagnostic performance of late-night UFCCR was superior to 24-h UFC. These results suggest that examination of late-night UFCCR might be a useful test for SCS diagnosis.

Our report is consistent with a previous report indicating that patients with SCS had higher late-night UFCCR.[21] Although only 4 SCS patients were included, that study found no significant correlation between MSC and late-night urinary cortisol levels.[21] The urinary samples in the previous report were collected at 11:00 p.m. without urine elimination taking place at 9:00 p.m., and for that reason, the samples did not reflect MSC levels. In contrast to the previous report, our study collected 2-h urinary samples after urine elimination at 9:00 p.m. Thus, the late-night UFCCR in our cases might more accurately reflect MSC levels.

Assessment of late-night UFCCR would be one of the simple and convenient methods among the many endocrinological assessments available for use, because patients can accurately collect urine samples on their own without requiring hospitalization especially compared to 24-h UFC. In cortisol overproduction state such as overt Cushing's syndrome, 24-h UFC displays very good sensitivity and is included in diagnostic criteria of Cushing's syndrome.[8,9,22] However, collection of all the urine over 24-h is cumbersome and potentially unreliable. Additionally, 24-h UFC may not reliably reveal a slight cortisol excess. Twenty-four-hour UFC therefore should not be considered an adequate screening test for SCS and should be used in combination with other tests.[6,8,10] In fact, only 1 patient with SCS showed above the

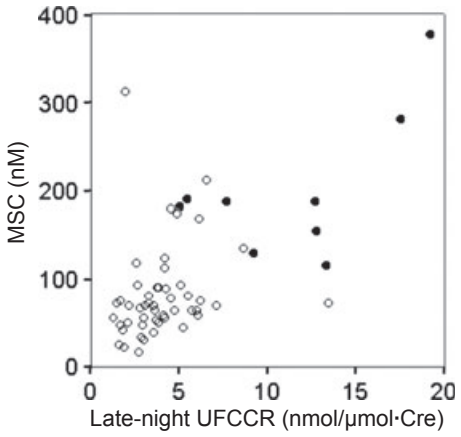

Fig. 2 Correlation between late-night urinary free cortisol to creatinine ratio (UFCCR) and midnight serum cortisol (MSC) levels. Spearman's correlation coefficient between late-night UFCCR and MSC levels was 0·556, indicating significant correlation ($P < 0·001$). Open circles: nonfunctioning adenoma ($n = 49$); closed circles: subclinical Cushing's syndrome ($n = 9$).

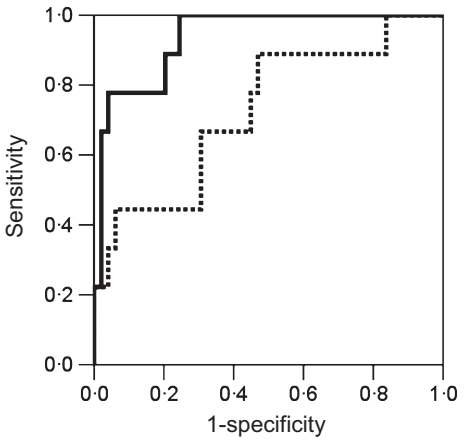

Fig. 3 The areas under the receiver operating characteristic (ROC) curves of endocrinological tests for diagnosis of subclinical Cushing's syndrome. The areas under the ROC curves of late-night urinary free cortisol to creatinine ratio (UFCCR) and 24-h urinary free cortisol (UFC) were 0·937 (0·865–1·008) and 0·726 (0·874–0·999), respectively. Solid line: late-night UFCCR; dotted line: 24-h UFC.

upper limit of 24-h UFC normal values in this study. In contrast, late-night UFCCR values were significantly higher in patients with SCS than in patients with NF and showed good sensitivity and specificity compared to 24-h UFC. Therefore, late-night UFCCR measurement might be a simple procedure and a useful test for diagnosis of SCS compared to 24-h UFC.

Salivary cortisol also can be collected without hospitalization, similar to the case of late-night UFCCR. Evidence of salivary cortisol levels being effective in SCS diagnosis has accumulated in recent years,[23,24] and the measurement represents a simple and convenient method for the assessment of cortisol production. Although there were 14 subjects in our study with less than the detection limit of UFC concentration between 9:00 p.m. and 11:00 p.m., all were diagnosed as having NF. UFC concentrations could be measurable in all patients with SCS. Late-night UFCCR as well as salivary cortisol might be easily measured without stress of pain and useful to assess the cortisol levels and diagnose SCS. Salivary cortisol cannot be applied to patients with gingivitis or Sjögren's syndrome because of bleeding risk or insufficient quantity of saliva.[25] Thus, late-night UFCCR might be an alternative to salivary cortisol in those patients.

Glucocorticoid increases hepatic glucose production, which induces adipose lipolysis, resulting in an increase in nonesterified fatty acids and triglycerides.[5] In addition, the excess circulating cortisol binds to mineralocorticoid receptor in the heart, blood vessels and kidneys.[13,14] Thus, SCS, which is defined as a state of cortisol excess, leads to metabolic disorders such as hypertension, dyslipidaemia and/or glucose intolerance.[3–5] Furthermore, an increase in the number of concomitant metabolic disorders is associated with an increase in the degree or severity of the SCS.[26] In this study, blood pressure, plasma glucose levels and serum lipid levels did not significantly differ between the two groups. Patients already undergoing treatment with antihypertensive drugs, antihyperlipidaemic drugs and/or hypoglycaemic drugs were included in our investigation. Such medication use might have affected the metabolism findings derived from this study.

Our study suffers from several limitations. First, it is necessary to consider the effect of renal function when late-night UFCCR is evaluated. Because renal impairment will strongly affect rate of cortisol excretion in urine,[27] we excluded patients with 24-h Ccr of less than 20 ml/min. Second, we measured late-night UFCCR in all patients only once. Therefore, we did not observe the individual day-to-day variation seen in the late-night UFCCR. Repeated measurements may be needed to confirm the usefulness of late-night UFCCR. Finally, our study also included a total of nine SCS patients. To determine whether late-night UFCCR is actually useful in SCS screening, the presented findings must be confirmed in a larger number of study subjects.

In conclusion, this study found that patients with subclinical Cushing's syndrome showed significantly higher late-night urinary free cortisol to creatinine ratio values than those with non-functioning adenoma, and late-night urinary free cortisol to creatinine ratio was significantly correlated with other endocrinological findings showing autonomous cortisol production. Diagnostic performance for subclinical Cushing's syndrome of late-night urinary free cortisol to creatinine ratio was superior to 24-h urinary free cortisol. We therefore suggest that the measurement of late-night urinary free cortisol to creatinine ratio represents one of the simple and useful tests for subclinical Cushing's syndrome diagnosis in patients with adrenocortical adenoma.

Disclosure

The authors have nothing to disclose.

References

1 Chiodini, I. (2011) Clinical review: diagnosis and treatment of subclinical hypercortisolism. *Journal of Clinical Endocrinology and Metabolism*, **96**, 1223–1236.

2 Emral, R., Uysal, A.R., Asik, M. *et al.* (2003) Prevalence of subclinical Cushing's syndrome in 70 patients with adrenal incidentaloma: clinical, biochemical and surgical outcomes. *Endocrine Journal*, **50**, 399–408.
3 Rossi, R., Tauchmanova, L., Luciano, A. *et al.* (2000) Subclinical Cushing's syndrome in patients with adrenal incidentaloma: clinical and biochemical features. *Journal of Clinical Endocrinology and Metabolism*, **85**, 1440–1448.
4 Di Dalmazi, G., Vicennati, V., Rinaldi, E. *et al.* (2012) Progressively increased patterns of subclinical cortisol hypersecretion in adrenal incidentalomas differently predict major metabolic and cardiovascular outcomes: a large cross-sectional study. *European Journal of Endocrinology*, **166**, 669–677.
5 Giordano, R., Guaraldi, F., Berardelli, R. *et al.* (2012) Glucose metabolism in patients with subclinical Cushing's syndrome. *Endocrine*, **41**, 415–423.
6 Valli, N., Catargi, B., Ronci, N. *et al.* (2001) Biochemical screening for subclinical cortisol-secreting adenomas amongst adrenal incidentalomas. *European Journal of Endocrinology*, **144**, 401–408.
7 Suda, T. (1997) Preclinical Cushing's syndrome and adrenocorticotropic hormone-independent bilateral adrenocortical macronodular hyperplasia. *Internal Medicine*, **36**, 601–602.
8 Nieman, L.K., Biller, B.M., Findling, J.W. *et al.* (2008) The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. *Journal of Clinical Endocrinology and Metabolism*, **93**, 1526–1540.
9 (2002) NIH state-of-the-science statement on management of the clinically inapparent adrenal mass ("incidentaloma"). *NIH consensus and state-of-the-science statements*, **19**,1–25.
10 De Leo, M., Cozzolino, A., Colao, A. *et al.* (2012) Subclinical Cushing's syndrome. *Best Practice and Research. Clinical Endocrinology Metabolism*, **26**, 497–505.
11 Mantero, F., Terzolo, M., Arnaldi, G. *et al.* (2000) A survey on adrenal incidentaloma in Italy. Study Group on Adrenal Tumors of the Italian Society of Endocrinology. *Journal of Clinical Endocrinology and Metabolism*, **85**, 637–644.
12 Oki, K., Yamane, K., Nakanishi, S. *et al.* (2012) Influence of adrenal subclinical hypercortisolism on hypertension in patients with adrenal incidentaloma. *Experimental and Clinical Endocrinology & Diabetes*, **120**, 244–247.
13 Newell-Price, J., Trainer, P., Perry, L. *et al.* (1995) A single sleeping midnight cortisol has 100% sensitivity for the diagnosis of Cushing's syndrome. *Clinical Endocrinology*, **43**, 545–550.
14 Papanicolaou, D.A., Yanovski, J.A., Cutler, G.B. Jr *et al.* (1998) A single midnight serum cortisol measurement distinguishes Cushing's syndrome from pseudo-Cushing states. *Journal of Clinical Endocrinology and Metabolism*, **83**, 1163–1167.
15 Köbberling, J. & von zur Mühlen, A. (1974) The circadian rhythm of free cortisol determined by urine sampling at two-hour intervals in normal subjects and in patients with severe obesity or Cushing's syndrome. *Journal of Clinical Endocrinology and Metabolism*, **38**, 313–319.
16 Burch, W. (2011) Using bedtime (PM) and early morning (AM) urine cortisol/creatinine ratios to evaluate pituitary-adrenal function in an office practice. *Endocrine Practice*, **17**, 591–597.
17 Kong, W.M., Alaghband-Zadeh, J., Jones, J. *et al.* (1999) The midnight to morning urinary cortisol increment is an accurate, noninvasive method for assessment of the hypothalamic-pituitary-adrenal axis. *Journal of Clinical Endocrinology and Metabolism*, **84**, 3093–3098.
18 Zeiger, M.A., Siegelman, S.S. & Hamrahian, A.H. (2011) Medical and surgical evaluation and treatment of adrenal incidentalomas. *Journal of Clinical Endocrinology and Metabolism*, **96**, 2004–2015.
19 Outwater, E.K., Siegelman, E.S., Huang, A.B. *et al.* (1996) Adrenal masses: correlation between CT attenuation value and chemical shift ratio at MR imaging with in-phase and opposed-phase sequences. *Radiology*, **200**, 749–752.
20 International Expert Committee. (2009) International expert committee report on the role of the A1C assay in the diagnosis of diabetes. *Diabetes Care*, **32**, 1327–1334.
21 Sakihara, S., Kageyama, K., Oki, Y. *et al.* (2010) Evaluation of plasma, salivary, and urinary cortisol levels for diagnosis of Cushing's syndrome. *Endocrine Journal*, **57**, 331–337.
22 Barrou, Z., Guiban, D., Maroufi, A. *et al.* (1996) Overnight dexamethasone suppression test: comparison of plasma and salivary cortisol measurement for the screening of Cushing's syndrome. *European Journal of Endocrinology*, **134**, 93–96.
23 Nunes, M.L., Vattaut, S., Corcuff, J.B. *et al.* (2009) Late-night salivary cortisol for diagnosis of overt and subclinical Cushing's syndrome in hospitalized and ambulatory patients. *Journal of Clinical Endocrinology and Metabolism*, **94**, 456–462.
24 Sereg, M., Toke, J., Patócs, A. *et al.* (2011) Diagnostic performance of salivary cortisol and serum osteocalcin measurements in patients with overt and subclinical Cushing's syndrome. *Steroids*, **76**, 38–42.
25 Yaneva, M., Mosnier-Pudar, H., Dugué, M.A. *et al.* (2004) Midnight salivary cortisol for the initial diagnosis of Cushing's syndrome of various causes. *Journal of Clinical Endocrinology and Metabolism*, **89**, 3345–3351.
26 Morelli, V., Masserini, B., Salcuni, A.S. *et al.* (2010) Subclinical hypercortisolism: correlation between biochemical diagnostic criteria and clinical aspects. *Clinical Endocrinology*, **73**, 161–166.
27 Chan, K.C., Lit, L.C., Law, E.L. *et al.* (2004) Diminished urinary free cortisol excretion in patients with moderate and severe renal impairment. *Clinical Chemistry*, **50**, 757–759.