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Original Research

Monitoring adrenal insufficiency through salivary steroids: a pilot study

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Abstract

Background: Various glucocorticoid replacement therapies (GRTs) are available for adrenal insufficiency (AI). However, their effectiveness in restoring glucocorticoid rhythm and exposure lacks adequate biochemical markers. We described the diurnal salivary cortisol (SalF) and cortisone (SalE) rhythm among different GRTs and analysed the associations between saliva-derived parameters and life quality questionnaires.

Methods: Control subjects (CSs, $n = 28$) and AI patients receiving hydrocortisone (HC, $n = 9$), cortisone acetate (CA, $n = 23$), and dual-release hydrocortisone once (DRHC-od, $n = 10$) and twice a day (DRHC-td, $n = 6$) collected 9 saliva samples from 07:00 to 23:00. Patients compiled Pittsburgh Sleep Quality Index, Hospital Anxiety and Depression Scale, and Addison disease-specific quality-of-life questionnaires. SalE and SalF were measured by liquid chromatography-mass spectrometry. Exposure was monitored using SalE for HC and DRHC and SalF for CA. Area under the curve (AUC) was computed. Different GRTs were compared by *Z*-scores calculated from saliva-derived parameters. Questionnaire results predictors were evaluated with multiple regression analysis.

Results: Compared with controls, all GRTs resulted in glucocorticoid overexposure in the morning. Hydrocortisone, CA, and DRHC-td caused overexposure also in afternoon and evening. Compared with other treatments, CA determined increased *Z*-score-07:00 ($P < .001$), DRHC-td determined increased *Z*-score-$AUC_{07:00\rightarrow14:00}$ ($P = .007$), and DRHC-od induced lower *Z*-score-$AUC_{14:00\rightarrow23:00}$ ($P = .015$). *Z*-scores-$AUC_{14:00\rightarrow16:00} \geq .619$ best predicted questionnaire scores.

Conclusions: None of the GRTs mimics normal glucocorticoid rhythmicity and exposure. SalE, SalF, and *Z*-score may be useful markers for monitoring and comparing different GRTs. Excess glucocorticoid in early afternoon best associated with depressive symptoms and worse life and sleep quality.

Keywords: adrenal insufficiency, glucocorticoid replacement therapy, salivary steroids, life and sleep quality, anxiety and depression

Significance

In the context of adrenal insufficiency, regardless of its aetiology, our work provided the first real-life comparison of diurnal glucocorticoid exposure between several replacement therapies by non-invasive salivary steroids monitoring. We exploited the high performance of liquid chromatography-tandem mass spectrometry for salivary cortisol and cortisone measurement and provided a new oral-contamination-free system thanks to the *Z*-score, which allowed the comparison between various glucocorticoid replacement therapies. We also described associations between high glucocorticoid exposure in the afternoon, worse sleep quality, and more anxiety-depressive symptoms. Our study enlightened the utility of non-invasive salivary steroids monitoring in adrenal insufficiency to describe diurnal differences between available replacement therapies and to prevent psychological disruption, although confirmatory studies are needed.

Introduction

Adrenal insufficiency (AI) is a chronic disease characterized by deficient hormone production by adrenal cortex, with tertiary AI as its major cause.[1-3] Adrenal insufficiency treatment requires life-long glucocorticoid replacement therapy (GRT) with prednisolone, hydrocortisone (HC), cortisone acetate (CA), or dual-release hydrocortisone (DRHC).[4-6] Compared with HC and CA, DRHC provides cortisol rhythm and exposure more similar to the physiological secretion. However, none of the current available treatments can reliably mimic the normal cortisol rhythmicity. Both overexposure and underexposure to glucocorticoids in patients with AI have

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been associated with increased risk of adrenal crisis and cardiometabolic consequences.[4,6-10] Moreover, in clinical settings, the management of AI is mostly based on clinical grounds as there is no available routine laboratory marker for monitoring GRT effectiveness in restoring glucocorticoid rhythm and exposure.[5,11,12] To this regard, the daylong measurement of salivary cortisol (SalF) and cortisone (SalE) has been proposed as a non-invasive approach for GRT management[10,13-18] and applied in a few clinical studies.[19-22] These studies evaluated glucocorticoid exposure with SalF and/or SalE in basal conditions, after GRT modifications and after shifting from HC or CA to once-a-day DRHC (DRHC-od).[19-22] Studies addressing the comparison of glucocorticoid exposure and rhythm among various GRTs have still not been performed.

Recent studies showed that alteration of glucocorticoid circadian rhythm typical of AI and other disease can impact life quality at many levels.[23,24] However, a previous study did not find any association between salivary glucocorticoid levels and life quality in AI.[21] Therefore, it is not clear how much psychological sequelae of AI patients depend on dysregulation of glucocorticoid balance in defined moments of the day, and if this dysregulation can be measured.

The primary aim is to analyse daily SalF and SalE levels and rhythm in patients under GRT with different drugs and schemes, regardless of AI aetiology. The secondary aim is to find parameters derived from diurnal SalF and SalE profiles associated with quality of life and sleep and with symptoms of anxiety and depression.

Methods

Patients

The study was approved by the Ethical Committee of the CE-AVEC (213/2015/O/Tess). All subjects gave written informed consent. We enrolled 48 patients with AI (32 primary AI [causes: 26 autoimmune adrenalitis, 5 previous bilateral adrenalectomy, and 1 previous prolonged mitotane therapy] and 16 secondary AI [causes: 9 previous pituitary or adrenal Cushing syndrome and 7 primary or secondary pituitary insufficiency]) referred to the Endocrinology and Diabetes Prevention and Care Unit of Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) S. Orsola Polyclinic of Bologna (Italy), between 2020 and 2022. Glucocorticoid replacement therapy type and dose were assigned by expert endocrinologist following guidelines criteria.[5] Inclusion criteria were age $\geq$ 18 years, no assumption of drugs interfering with steroid hormone production, normal thyroid function (based on age and/or aetiology), and duration of AI for 6 months or more. Patients were divided in 4 groups according to the type of GRT: HC twice or three times a day (group "HC," $n = 9$), DRHC twice a day (group "DRHC-td," $n = 6$), CA twice or three times a day (group "CA," $n = 23$), and DRHC-od ($n = 10$). Patients belonging to the DRHC-td were previously under DRHC-od regimen; however, after experiencing fatigue and under-treatment symptoms in late afternoon, they split the DRHC from od to td, with the larger dose in the morning (25-20 mg) and the smaller in the afternoon (5 mg). All patients with mineralocorticoid insufficiency were treated with fludrocortisone. None of the patients were treated with growth hormone.

We also enrolled 28 control subjects (CSs) matching the following inclusion criteria: age $\geq$ 18 years, complete sexual development, and regular menses in females in reproductive age. Exclusion criteria were any disease (except for compensated hypothyroidism) or medication (except for levothyroxine replacement treatment), drug or alcohol abuse, pregnancy, lactation, sleep disturbances, and/or unpreserved circadian rhythm and gingivitis.

All the procedures conducted during this study were performed in compliance with the Declaration of Helsinki.

Clinical, biochemical, and radiological evaluation

Adrenal insufficiency aetiology was retrieved from patient records. Adrenal insufficiency duration was calculated in years from diagnosis date to saliva collection date. Time span of ongoing GRT scheme was estimated in weeks from the date of the prescription of the last scheme to saliva collection date. We collected anthropometric data like body mass index (BMI), waist and hip circumference, and body surface area (Du Bois formula: $0.007184 * \text{Height}^{0.725} * \text{Weight}^{0.425}$) for all participants.[25] We also registered patient biochemical data (glycolipid metabolism, kidney function, electrolytes balance, thyroid function, and bone metabolism) from fasting blood sample analysis conducted the day after saliva collection, according to previously described procedures.[26] Bone radiological data from lumbar and femoral neck dual-energy X-ray absorptiometry and vertebral morphology by dorsal-lumbar spine X-ray conducted at IRCCS Sant'Orsola Polyclinic were only considered in patients with AI duration longer than 4 years, if performed within 2 years before saliva collection. Low bone density was defined according to the current guidelines.[27] Hydrocortisone-equivalent dose for CA was calculated. Subsequently, HC-equivalent dose per body surface area (Eqdose) and weight were calculated for each patient.

Questionnaires

Patients performed the following questionnaires: the Adrenal insufficiency-specific Quality of Life (AddiQoL),[28] the Pittsburgh Sleep Quality Index (PSQI),[29] and the Hospital Anxiety and Depression Scale (HADS).[30] The AddiQoL has 3 main subsections named fatigue, emotions, and symptoms; score ranges from 0 to 120, and increasing results reflect better life quality; however, no reference limits are available.[28] The PSQI ranges from 0 to 21; high values reflect poor sleep quality and a score from 0 to 5 is considered normal.[29] The HADS ranges from 0 to 42, with 2 subsections: anxiety and depression. Symptoms of anxiety and depression are associated with increasing values and a score from 0 to 7 is considered normal.[30] The results of the questionnaires were expressed as continuous scores. The PSQI and HADS were also presented as categorical dichotomic variables (normal vs elevated).

Saliva collection and hormonal assay

All subjects received written instructions on saliva collection. Samples were collected by using Salivette Cortisol© tubes (Sarstedt, Nümbrecth, Germany). Patients were instructed to hold the cotton swab under the tongue for at least 3 min without touching or chewing and to avoid eating, drinking, smoking, or brushing teeth in the 30 min before. The collection day was scheduled on an ordinary day at home, without altering their normal daily activities, avoiding any stress related to the hospital setting. All patients took the first GRT dose at

07.00, the second between 12:00 and 16:00, and the third between 16:00 and 18:00 AM, according to medical instructions. Saliva samples were collected at 07:00 (awakening time), 07:30, 10:00, 12:30 (before lunch), 14:00, 16:00, 19:30 (before dinner), 21:00, and 23:00 (bedtime). At each time point, sampling was performed immediately before or 30 min or more after drug intake. Samples were stored in home freezer and delivered to the laboratory the day after. Salivettes were centrifuged according to the manufacturer's instructions, and samples were stored at −80 °C until assayed. SalF and SalE were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to a previously validated *in-house* method.[17]

Statistical analysis

Three patients (1 DRHC-td, 1 CA, and 1 DRHC-od) and 1 control subject missed the saliva sample on 1 time point within 07:30 and 21:00. Such missing values were computed through linear interpolation as previously reported.[31] Two patients (both CA) and 1 control subject missed the saliva sample at 07:00 or 23:00. These missing values were replaced with the mean value within the same GRT group at the same time point. The area under the curve (AUC) for SalF and SalE was determined with the trapezoidal rule including all consecutive saliva samples from 07:00 to 23:00 ($AUC_{07:00\rightarrow23:00}$), from 07:00 to 14:00 ($AUC_{07:00\rightarrow14:00}$), from 12:30 to 16:00 ($AUC_{12:30\rightarrow16:00}$), and from 14:00 to 23:00 ($AUC_{14:00\rightarrow23:00}$). Because of drug contamination in the buccal mucosa after GRT intake,[10] the measurements of SalF in HC, DRHC-td, and DRHC-od and of SalE in CA were not reliably reflecting the circulating levels. Therefore, we considered SalF in patients under CA treatment and SalE in patients under HC, DRHC-td, and DRHC-od treatment as indicators of glucocorticoid levels, since they both have been demonstrated to be strongly correlated to serum cortisol.[10,13,17,18,32,33] Moreover, previous studies in patients receiving HC medications did not highlight the saturation of 11β-hydroxysteroid dehydrogenase type 2 activity in the parotid cells.[13] In addition, we calculated the *Z*-scores of glucocorticoid levels and AUC including in the reference population both patients and control subjects. *Z*-score indicates how many SDs a value is away from the group mean, and, since it is dimensionless, it could be compared between groups of patients with different GRTs. Categorical variables were displayed as absolute numbers and proportions and compared with Pearson's χ^2. Continuous variables were log-10 transformed when normality of distribution was not satisfied and reported as mean ± SD. For SalF and SalE, a normal distribution was not achieved even after transformation; therefore, these variables were reported as median and interquartile ranges. Continuous variables were compared with Student *t*-test, or ANOVA, or Mann–Witney *U* test and Kruskal–Wallis where appropriate. Friedman test was used to evaluate the presence of daily fluctuation in salivary glucocorticoids, whereas the Wilcoxon tests were used to compare values at any time with values at 07:00 AM. Two-way ANOVA with Tukey post hoc analysis was performed to examine the effect of different GRTs and time of sampling on single *Z*-scores. Area under the curve *Z*-scores were compared with ANOVA with contrast. Stepwise multiple linear regression was performed including AddiQoL, HADS, and PSQI scores, overall or main subsections as dependent variables and disease duration, EqDose, age, BMI, ongoing GRT scheme duration, and glucocorticoid *Z*-scores as independent variables. Forward multiple logistic regression was applied including dichotomic HADS and PSQI as dependent variables and EqDose, age, GRT type and scheme duration, AI aetiology and duration, BMI, ongoing, and glucocorticoid *Z*-scores as independent variables. Significant predictors defined by the logistic regressions were analysed by the receiver operating characteristic (ROC) curve analysis to identify the best predicting value. Statistics was performed by SPSS 22.0 (IBM©, Chicago, IL, USA). *P* values < .05 were considered significant.

Results

General and clinical features of the cohort

General and anthropometric data are displayed in Table 1. Control subjects were younger than DRHC-td, CA, and DRHC-od, and CA patients were older than those under DRHC-od ($P < .001$). Body mass index was similar among GRT-groups but larger in HC and CA compared to controls ($P = .020$). Waist circumference was larger in HC, DRHC-td, and CA than controls and in CA compared with DRHC-od ($P < .001$). Patients under CA displayed higher Eqdose than those taking HC ($P = .041$), while dose per weight was not different among groups ($P = .146$) (Table 2).

Table 1. Population general and anthropometric characteristics.

	Hydrocortisone (HC, $n = 9$)	Dual-release hydrocortisone twice a day (DRHC-td, $n = 6$)	Cortisone acetate (CA, $n = 23$)	Dual-release hydrocortisone once a day (DRHC-od, $n = 10$)	Control subjects ($n = 28$)	*P*
Age (years)	42.4 ± 20.8	47.1 ± 14.3[b]	57.1 ± 11.5[a,b]	46.7 ± 10.8[b]	30.0 ± 11.23	**<.001**
Body Mass Index (kg/m^2)	26.4 ± 3.78[b]	23.4 ± 1.4	26.3 ± 4.4[b]	24.2 ± 4.6	22.9 ± 3.4	**.020**
Waist circumference (cm)	89.8 ± 9.1[b]	86.6 ± 5.1[b]	96.2 ± 11.8[a,b]	82.3 ± 9.5	76.7 ± 8.2	**<.001**
Hip circumference (cm)	99.3 ± 4.7	96.4 ± 2.7	99.3 ± 21.9	94.4 ± 8.9	94.5 ± 6.9	.223
Female (%)	5 (55.6)	2 (33.3)	13 (56.5)	5 (50.0)	14 (50.0)	.892

Categorical data were presented as proportion (%); *P* value was estimated through Pearson's χ^2.
Continuous data were presented as mean ± SD; *P* value was estimated through ANOVA.
Significant differences are highlighted in bold.
[a]$P < .05$ vs DRHC-od.
[b]$P < .05$ vs controls.

Table 2. Patients with adrenal insufficiency: GRT treatment information, questionnaire results, comorbidities, and biochemistry.

	Hydrocortisone (HC, $n = 9$)	Dual-release hydrocortisone twice a day (DRHC-td, $n = 6$)	Cortisone acetate (CA, $n = 23$)	Dual-release hydrocortisone once a day (DRHC-od, $n = 10$)	P
GRT treatment					
Adrenal insufficiency aetiology (primary/secondary)	9/0[a]	5/1	11/12	7/3	**.030**
Adrenal insufficiency duration (years)	14.7 ± 16.0[a]	15.8 ± 11.1[a]	7.0 ± 12.8	10.2 ± 6.0[a]	**.001**
HC-equivalent dose (mg/m^2 per day)	12.5 ± 4.1[a]	15.3 ± 1.1	16.1 ± 3.3	14.2 ± 2.5	**.041**
HC-equivalent dose (mg/kg per day)	0.32 ± 0.12	0.40 ± 0.4	0.41 ± 0.9	0.38 ± 0.11	.146
Ongoing GRT scheme duration (weeks)	131 ± 109	201 ± 148	146 ± 231	173 ± 110	.288
Comorbidities					
Previous adrenal Cushing's syndrome, *n* (%)	1 (11.1)	1 (16.7)	10 (43.5)	1 (10.0)	.107
Insufficient RAAS, *n* (%)	9 (100.0)[a]	5 (83.3)	10 (43.5)	7 (70.0)	**.015**
Hypertension, *n* (%)	0 (0.0)[a]	0 (0.0)[a]	11 (47.8)	0 (0.0)[a]	**.001**
Dyslipidaemia, *n* (%)	5 (55.6)	1 (16.7)	13 (59.1)	5 (50.0)	.323
Diabetes, *n* (%)	1 (11.1)	0 (0.0)	4 (17.4)	0 (0.0)	.382
Reduced bone density/osteopenia, *n* (%)	1 (20.0)	2 (50.0)	8 (53.3)	6 (60.0)	.583
Osteoporosis/severe osteoporosis, *n* (%)	3 (60.0)	2 (50.0)	4 (26.7)	4 (40.0)	.515
Ischaemic heath disease or stroke, *n* (%)	0 (0.0)	0 (0.0)	2 (8.7)	0 (0.0)	.519
Biochemistry [laboratory reference intervals]					
Aspartate transaminase (IU/L) [<35]	23.7 ± 7.5	19.6 ± 5.4	23.0 ± 8.6	19.3 ± 4.1	.495
Alanine transaminase (IU/L) [<35]	21.6 ± 7.5	13.8 ± 4.3	19.5 ± 12.8	16.1 ± 4.9	.480
Creatinine (mg/dL) [0.5-1.2]	0.82 ± 0.24	0.91 ± 0.18	0.88 ± 0.29	.88 ± .14	.894
Glomerular filtrate (mL/min) [>60]	99.6 ± 27.3	92.4 ± 16.6	86.3 ± 18.8	90.8 ± 17.0	.447
Fasting glycaemia (mg/dL) [60-100]	84.1 ± 19.9	74.3 ± 16.0	80.6 ± 10.8	78.9 ± 12.1	.664
Glycosylated haemoglobin (mmol/mol) [20-42]	39.6 ± 13.6	35.0 ± 4.6	36.4 ± 3.4	34.3 ± 6.2	.542
Fasting insulin (mIU/mL) [1.9-23.0]	17.1 ± 22.1	4.2 ± 1.0	8.5 ± 5.9	5.7 ± 2.7	.148
Total cholesterol (mg/dL) [<200]	184.6 ± 33.1	202.2 ± 34.3	197.3 ± 54.4	212.0 ± 45.8	.720
High density lipoprotein (mg/dL) [female: >45; male: >35]	51.9 ± 6.8	66.2 ± 16.8	61.8 ± 20.4	59.0 ± 14.1	.482
Low-density lipoprotein (mg/dL) [<116]	106.6 ± 28.4	120.6 ± 29.4	109.0 ± 43.6	131.6 ± 31.6	.483
Triglycerides [<150]	137.0 ± 44.8	95.6 ± 20.4	139.6 ± 80.4	136.0 ± 81.7	.665
Blood Calcium (mg/dL) [8.6-10.5]	9.40 ± .52	9.18 ± .43	9.25 ± .34	9.55 ± 0.49	.295
25-hydroxy-vitamin D (ng/mL) [>12.0]	27.9 ± 8.5	43.3 ± 11.6	27.0 ± 13.9	34.8 ± 15.6	.112
C-terminal telopeptide (ng/mL) [female: pre-menopause .112–.738, post-menopause .142-1.351; male: .115–.748]	0.775 ± 0.461	0.253 ± 0.132	2.848 ± 7.840	0.439 ± 0.279	.273
Bone alkaline phosphatase (µg/L) [female pre-menopause 4.7-27.0, post-menopause 5.5-27.1; male 5.7-32.9]	20.3 ± 7.1	16.5 ± 6.2	18.5 ± 6.9	13.7 ± 5.4	.756
Thyrotropin (mIU/mL) [.25-4.50]	3.17 ± 2.63	2.74 ± 1.20	3.04 ± 3.29	2.41 ± 2.56	.334
Sodium (mmol/L) [136-145]	139 ± 3	142 ± 3	141 ± 3	141 ± 3	.506
Potassium (mmol/L) [3.5-5.3]	4.1 ± 0.3	4.0 ± 0.2	4.1 ± 0.4	4.2 ± 0.5	.708
Questionnaires					
AddiQoL—overall	84.1 ± 10.7	94.8 ± 10.9	85.5 ± 17.1	80.6 ± 11.1	.428
AddiQoL—fatigue	19.5 ± 4.3	23.5 ± 5.1	19.8 ± 7.2	20.4 ± 4.8	.694
AddiQoL—symptoms	27.9 ± 3.1	29.3 ± 4.2	27.3 ± 4.6	26.1 ± 4.2	.652
AddiQoL—emotions	22.34 ± 3.0	26.0 ± 1.6	24.3 ± 5.0	22.3 ± 3.2	.313
HADS—overall	7.0 ± 2.2	3.8 ± 3.4	10.6 ± 9.7	10.7 ± 5.0	.310
HADS—anxiety	4.6 ± 1.4	2.5 ± 1.9	5.8 ± 5.4	5.7 ± 2.3	.286
HADS—depression	2.4 ± 1.5	1.3 ± 1.5	4.8 ± 4.6	5.0 ± 3.7	.335
Altered HADS (score ≥8), *n* (%)	2 (28.6)	1 (25.0)	7 (58.3)	5 (83.3)	.154
PSQI	5.4 ± 2.8	5.3 ± 1.7	6.1 ± 2.8	8.3 ± 3.7	.350
Altered PSQI (score ≥6), *n* (%)	3 (42.9)	2 (50.0)	6 (50.0)	5 (83.3)	.471

Categorical data were presented as proportion (%); P value was estimated through Pearson's χ^2.
Continuous data were presented as mean ± SD; P value was estimated through ANOVA.
Significant differences are highlighted in bold.
AddiQoL, Adrenal insufficiency-specific Quality of Life; GRT, glucocorticoid replacement therapy; HADS, Hospital Anxiety and Depression Scale; Ongoing GRT scheme duration, weeks of actual GRT during salivary sampling; PSQI, Pittsburgh Sleep Quality Index; RAAS, renin–angiotensin–aldosterone system.
[a]$P < .05$ vs CA.

Salivary glucocorticoid levels and AUCs

SalF and SalE values at single time points and AUCs by groups are reported in Table S1. Figures 1-4 show salivary glucocorticoids profiles in each GRT groups vs controls. No differences in SalE or SalF levels were observed between 2 or 3 GRT administrations in patients under HC (Table S2) and CA (Table S3). Conversely, several differences were observed between DRHC-od and DRHC-td (Table S4). Differences between patients with primary and secondary AI were detected only at SalF-19.30 ($P = .019$; Table S5) and SalE-10.00 ($P = .047$; Table S6); hence, we decided not to separate patients with primary and secondary adrenal insufficiency.

SalE levels among patients taking HC, DRHC-td, DRHC-od, and controls were different at all time points except at 07:30 and 23:00 ($P < .001$-$.023$). SalE levels at 07:00 were markedly lower in patients than controls. Patients under

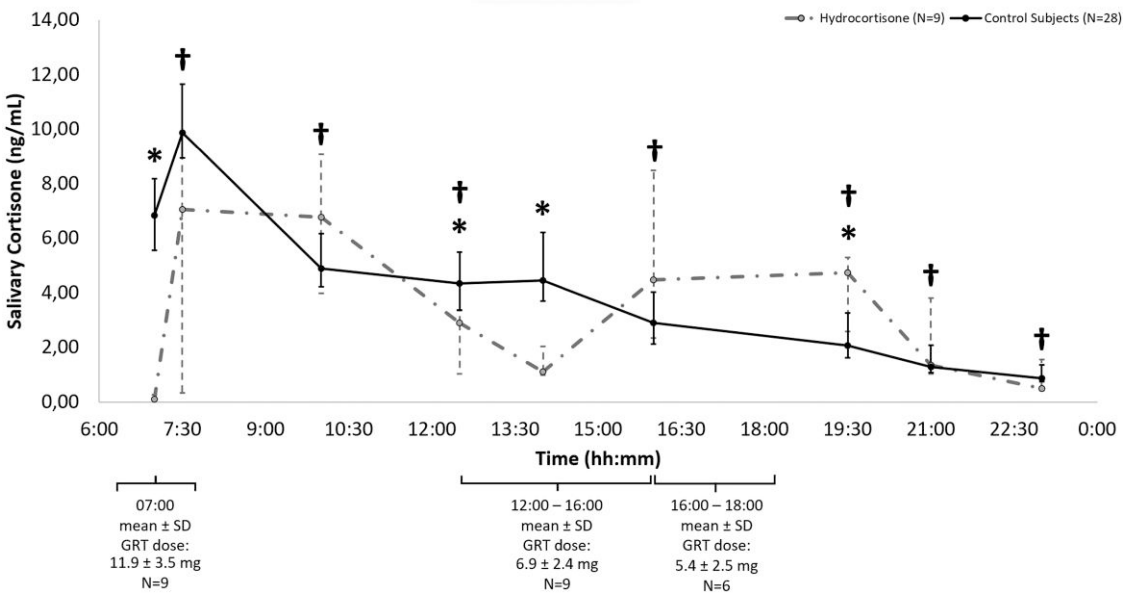

Figure 1. Salivary cortisone levels in patients under hydrocortisone vs controls.

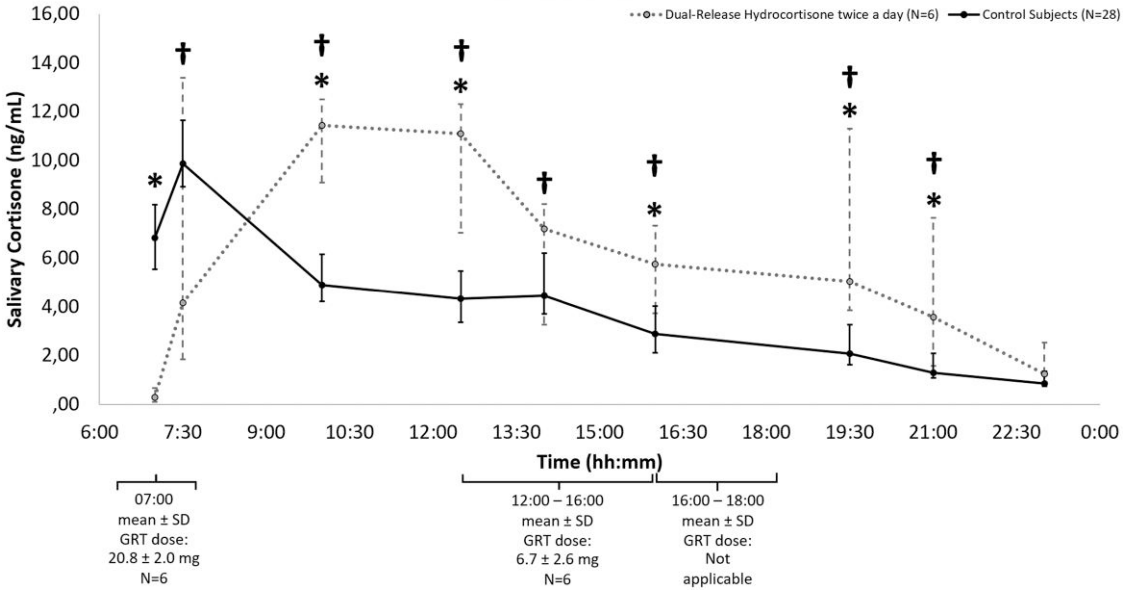

Figure 2. Salivary cortisone levels in patients under dual-release hydrocortisone twice a day vs controls.

DRHC showed higher exposure to glucocorticoids in the morning (10:00 and 12:30) than those taking HC and CS, whereas patients under HC had lower salivary glucocorticoid levels at 14:00 than other groups.

SalF levels in CA were higher than controls at 10:00, 19:30, and 21:00 ($P < .001$-$.001$) but lower at 07:00, 07:30, and 14:00 ($P < .001$-$.009$).

Friedman test detected significant salivary glucocorticoid fluctuation in each AI group (all $P < .001$). Results of Wilcoxon repeated measures comparisons are graphically reported in Figures 1-4. In HC group, all salivary samples differed from 07:00 ($P = .008$-$.049$), except for 14:00. In DRHC-td patients, all salivary samples differed from 07:00 ($P = .028$), except for 23:00. In CA patients, all salivary samples differed from 07:00 ($P < .001$-$.029$), except for 14:00, 21:00, and 23:00. In DRHC-od group, only salivary samples from 07:30 to 14:00 differed from 07:00 ($P = .005$-$.007$).

All AUC intervals are reported in Table S1. Area under the curve for SalE was different among the 4 groups, except for $AUC_{07:30\rightarrow10:00}$, $AUC_{14:00\rightarrow16:00}$, and $AUC_{21:00\rightarrow23:00}$ ($P < .001$-$.019$). $AUC_{16:00\rightarrow19:30}$ and $AUC_{19:30\rightarrow21:00}$ were lower in patients taking DRHC-od than other groups. Subjects under DRHC-td showed higher $AUC_{07:00\rightarrow23:00}$ and $AUC_{12:30\rightarrow16:00}$ compared with other groups. Area under the curve in the morning ($AUC_{07:00\rightarrow14:00}$) was lower in patients under HC than other groups.

Compared with controls, CA displayed higher AUC for SalF mostly in the afternoon ($AUC_{16:00\rightarrow19:30}$, $AUC_{19:30\rightarrow21:00}$, $AUC_{21:00\rightarrow23:00}$, $AUC_{14:00\rightarrow23:00}$, and $AUC_{07:00\rightarrow23:00}$; $P < .001$ for all).

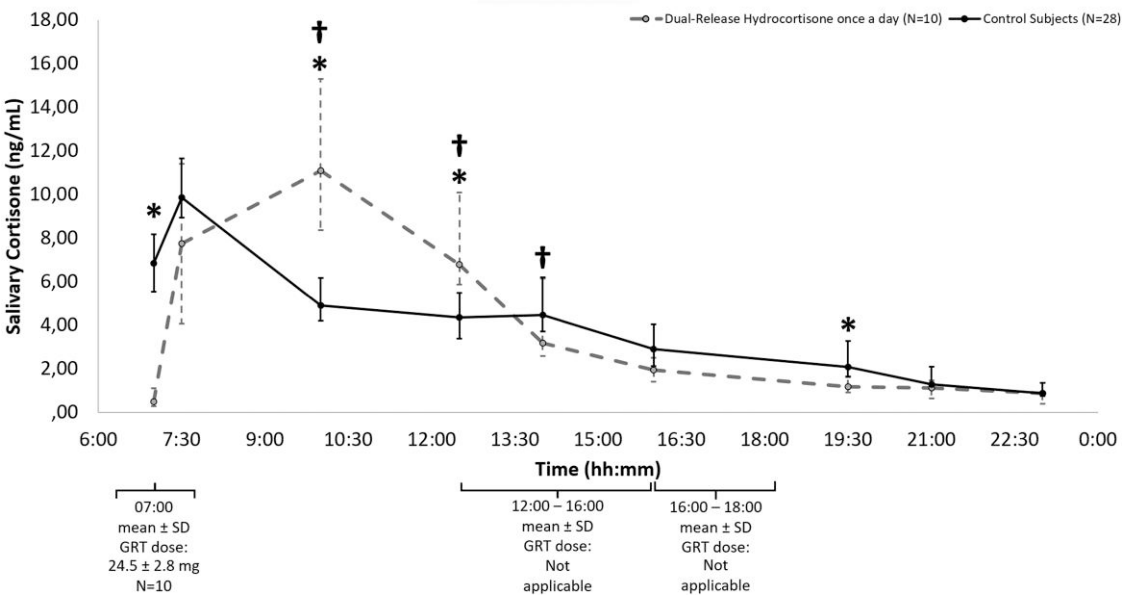

Figure 3. Salivary cortisone levels in patients under dual-release hydrocortisone once a day vs controls.

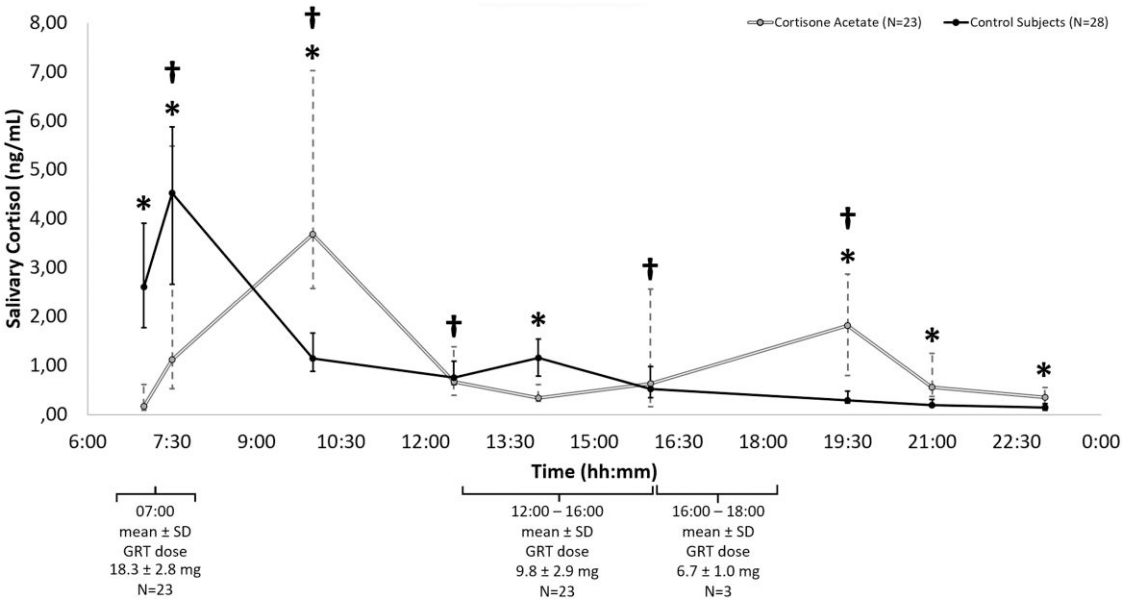

Figure 4. Salivary cortisol levels in patients under cortisone acetate vs controls.

Comparison of glucocorticoid *Z*-scores among GRT groups

Z-score values significantly varied along the day within each GRT group ($P < .001$-$.010$) (Table 3 and Figure 5). The 2-way ANOVA revealed significant interaction between the effect of different GRT and sampling time ($P = .026$) on *Z*-scores. *Z*-score at 07:00 was negative in all patients ($P < .001$-$.010$) and was significantly lower compared with 16:00 and 19:30 within patients under HC, to 10:00, 12:30, and 19:30 within subjects taking DRHC-td, to 10:00, 19:30, and 21:00 within patients under CA, and to 10.00 and 12:30 within patients taking DRHC-od (Table 3).

Concerning single time-point *Z*-scores, these were different among patients at 07:00, 12:30, and 19:30 ($P < .001$-$.047$). At 07:00, HC, DRHC-td, and DRHC-od presented larger negative *Z*-score compared with CA. At 12:30, HC showed larger negative *Z*-score compared with both DRHC groups. At 19.30, the negative *Z*-score by DRHC-od was lower than DRHC-td.

Considering *Z*-score-AUCs (Table 3), $AUC_{10:00\rightarrow12:30}$, $AUC_{12:30\rightarrow14:00}$, $AUC_{16:00\rightarrow19:30}$, $AUC_{19:30\rightarrow21:00}$, $AUC_{07:00\rightarrow14:00}$, and $AUC_{14:00\rightarrow23:00}$ were different among groups ($P = .003$-$.043$). Subjects taking HC presented a larger negative $AUC_{12:30\rightarrow14:00}$ than other patients. Patients under DRHC-od showed a larger negative $AUC_{16:00\rightarrow19:30}$ and $AUC_{14:00\rightarrow23:00}$ than other groups and a larger negative $AUC_{19:30\rightarrow21:00}$ compared with subjects under DRHC-td and CA. Patients taking HC presented larger negative $AUC_{07:00\rightarrow14:00}$ compared with those under DRHC.

Table 3. *Z*-score values by GRT groups.

	Hydrocortisone (HC, $n = 9$)	Dual-release hydrocortisone twice a day (DRHC-td, $n = 6$)	Cortisone acetate (CA, $n = 23$)	Dual-release hydrocortisone once a day (DRHC-od, $n = 10$)	*P*
Salivary sample *Z*-score					
07.00	−1.38 ± 0.28[c]	−1.55 ± 0.33[c]	−0.67 ± 0.49	−1.27 ± 0.41[c]	**<.001**
07.30	−0.68 ± 1.63	−0.32 ± 2.20	−0.12 ± 1.41	−0.33 ± 1.68	.847
10.00	0.39 ± 1.58	1.68 ± 1.04[g]	0.56 ± 1.01[h]	1.17 ± 1.34[h]	.124
12.30	−0.84 ± 0.65[d]	1.57 ± 1.22[a,c,g]	0.10 ± 1.34	0.91 ± 1.41[h]	**.002**
14.00	−0.74 ± 1.20	0.55 ± 1.20	−0.48 ± 0.64	−0.26 ± 1.09	.067
16.00	0.75 ± 1.65[f]	1.05 ± 1.05	0.30 ± 1.38	−0.56 ± 0.52	.064
19.30	0.86 ± 1.61[f]	1.39 ± 1.60[e,g]	0.70 ± 1.16[h]	−0.41 ± 1.19	**.047**
21.00	0.43 ± 1.63	1.22 ± 1.93	0.54 ± 1.33[h]	−0.37 ± 1.00	.173
23.00	−0.09 ± 1.26	0.77 ± 1.95	0.36 ± 1.45	−0.14 ± 0.90	.530
AUC Z-score					
07.00-07.30	−1.21 ± 1.00	−1.08 ± 1.59	−0.38 ± 1.22	−0.94 ± 1.20	.271
07.30-10.00	−0.41 ± 1.57	0.68 ± 1.95	0.25 ± 1.32	0.82 ± 1.45	.306
10.00-12.30	−0.26 ± 1.25[b]	1.84 ± 0.65	0.52 ± 1.05[b]	1.13 ± 1.37[a,b]	**.005**
12.30-14.00	−0.88 ± 0.96[b,d]	1.33 ± 1.26[c]	−0.25 ± 1.11	0.56 ± 1.43	**.003**
14.00-16.00	0.12 ± 1.59	0.93 ± 1.24	−0.002 ± 1.29	−0.46 ± 0.99	.237
16.00-19.30	0.94 ± 1.59[d]	1.50 ± 1.28[d]	0.67 ± 1.11[d]	−0.62 ± 1.00	**.006**
19.30-21.00	0.77 ± 1.66	1.48 ± 1.59[d]	0.69 ± 1.19[d]	−0.44 ± 1.22	**.043**
21.00-23.00	0.24 ± 1.54	1.09 ± 1.96	0.50 ± 1.37	−0.28 ± 0.89	.280
07.00-23.00	0.15 ± 1.50	1.64 ± 0.83	0.46 ± 1.17	0.56 ± 1.50	.167
07.00-14.00	−0.69 ± 1.18[b]	1.49 ± 0.93[c]	0.30 ± 1.25	0.94 ± 1.42[c]	**.007**
12.30-16.00	−0.36 ± 1.32	1.18 ± 1.24	−0.11 ± 1.15	0.09 ± 1.31	.103
14.00-23.00	0.76 ± 1.61[d]	1.50 ± 1.32[d]	0.62 ± 1.17[d]	−0.57 ± 1.05	**.015**

Continuous data were presented as mean ± SD; *P* value was estimated through 2-way-ANOVA with Tukey post hoc test for salivary sample *Z*-score and with ANOVA with contrast for AUC *Z*-score.
Significant differences are highlighted in bold.
AUC, area under the curve; CA, cortisone acetate; DRHC-od, dual-release hydrocortisone once a day; DRHC-td, dual-release hydrocortisone twice a day; HC, hydrocortisone.
[a]$P < .05$ vs HC.
[b]$P < .05$ vs DRHC-td.
[c]$P < .05$ vs CA.
[d]$P < .05$ vs DRHC-od.
[e]$P = .051$ vs DRHC-od.
[f]$P < .05$ vs 07.00 (HC).
[g]$P < .05$ vs 07.00 (DRHC-td).
[h]$P < .05$ vs 07.00 (CA).

Associations of psychometric scores with indicators of glucocorticoid exposure

We compared the *Z*-scores between patients with normal vs elevated PSQI and HADS results (results in Table 4). Patients with elevated PSQI score showed higher *Z*-scores-14:00 ($P = .012$), $AUC_{14:00\rightarrow16:00}$ ($P = .006$), and $AUC_{12:30\rightarrow16:00}$ ($P = .005$) compared with those with normal PSQI results.

Results of multivariate regression analysis are reported in Table 5. *Z*-score-16:00 and *Z*- score-$AUC_{14:00\rightarrow16:00}$ negatively predicted the overall AddiQol score, fatigue and emotions ($P = .003$–$.048$). Symptoms were positively predicted by *Z*-score-23:00 ($P = .044$). *Z*-score-16:00, *Z*-score-$AUC_{07:00\rightarrow07:30}$, and *Z*-score-$AUC_{14:00\rightarrow16:00}$ positively predicted overall HADS score and anxiety ($P = .007$–$.038$). Anxiety was negatively predicted by a model including *Z*-score-$AUC_{14:00\rightarrow16:00}$ and ongoing GRT scheme duration ($P = .015$). *Z*-score-16:00 and *Z*-score-$AUC_{14:00\rightarrow16:00}$ positively predicted depression ($P = .013$ and $P = .016$, respectively). *Z*-score-14:00, *Z*-score-$AUC_{14:00\rightarrow16:00}$, and *Z*-score-$AUC_{12:30\rightarrow16:00}$ positively predicted the overall PSQI score ($P = .005$–$.006$). Elevated PSQI was predicted by *Z*-score-$AUC_{14:00\rightarrow16:00}$ ($P = .021$) and *Z*-score-$AUC_{12:30\rightarrow16:00}$ ($P = .021$).

The ROC curve analysis showed that a value of .619 of *Z*-score-$AUC_{14:00\rightarrow16:00}$ provided the best sensitivity and specificity in predicting altered PSQI (sensitivity: 56.3%; specificity: 100.0%; AUC = 0.788; 95% CI, 0.623-0.954; $P = .009$). Subsequently, we estimated the cut-offs of $AUC_{14:00\rightarrow16:00}$ corresponding to .619 *Z*-score-$AUC_{14:00\rightarrow16:00}$ of cortisone in HC, DRHC-td, and DRHC-od groups (10.6 ng/mL/2 h, 10.9 ng/mL/2 h, and 9.5 ng/mL/2 h, respectively) and cortisol in CA group (3.2 ng/mL/2 h). Accordingly, 3 patients under HC (33.3%), 3 patients under DRHC-td (50.0%), 4 patients under CA (17.4%), and 2 patients under DRHC-od (20.0%) presented glucocorticoid $AUC_{14:00\rightarrow16:00}$ equal or larger than the above-mentioned cut-offs ($P = .365$). Afterwards, we analysed the distribution of AddiQoL overall and subsection scores, and the frequencies of altered HADS and PSQI according to values of glucocorticoid $AUC_{14:00\rightarrow16:00}$ below or above the defined cut-offs (Tables 6 and 7). Lower levels of AddiQol overall ($P = .002$), fatigue ($P = .003$), symptoms ($P = .047$), and emotions ($P = .015$) subdomain scores and higher proportion of altered PSQI ($P = .003$) and HADS ($P = .039$) were associated with glucocorticoid $AUC_{14:00\rightarrow16:00}$ values above .619 *Z*-score.

Discussion

This study assessed the differences in glucocorticoid rhythm and exposure among patients with AI undergoing different schemes of GRT. We attempted to identify indicators of under

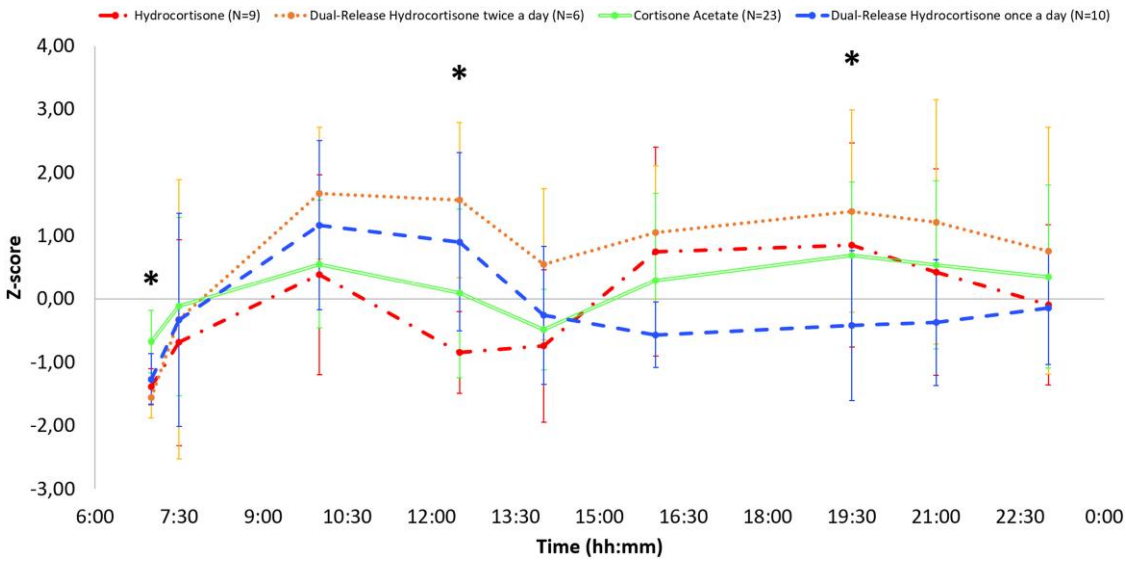

Figure 5. *Z*-score in patients with adrenal insufficiency.

Table 4. *Z*-score values by PSQI and HADS results.

	PSQI			HADS		
	Normal ($n = 13$)	Altered ($n = 16$)	*P*	Normal ($n = 14$)	Altered ($n = 15$)	*P*
Salivary sample *Z*-score						
07.00	−1.2 ± 0.41	−1.04 ± 0.52	.383	−1.28 ± 0.41	−1.01 ± 0.51	.125
07.30	−0.63 ± 1.2	0.05 ± 2.16	.321	−0.04 ± 1.96	−0.34 ± 1.71	.657
10.00	0.86 ± 1.13	1.14 ± 1.61	.608	0.95 ± 1.48	0.87 ± 1.17	.878
12.30	0.05 ± 1.91	0.56 ± 1.49	.422	0.26 ± 1.79	0.07 ± 1.19	.739
14.00	−0.85 ± 0.63	−0.01 ± 1.03	**.012**	−0.56 ± 0.97	−0.32 ± 0.85	.491
16.00	−0.1 ± 0.82	0.83 ± 1.58	.053	−0.06 ± 0.63	0.75 ± 1.77	.114
19.30	0.52 ± 0.99	0.32 ± 1.48	.673	0.52 ± 1	0.06 ± 1.36	.312
21.00	0.46 ± 0.94	0.22 ± 1.57	.635	0.3 ± 1.04	0.18 ± 1.48	.803
23.00	0.12 ± 0.8	−0.01 ± 1.32	.751	−0.12 ± 0.79	0.17 ± 1.34	.484
AUC *Z*-score						
07.00-07.30	−1.1 ± 0.94	−0.48 ± 1.67	.245	−0.72 ± 1.38	−0.74 ± 1.48	.970
07.30-10.00	0.04 ± 0.75	0.81 ± 2.1	.186	0.51 ± 1.84	0.32 ± 1.42	.761
10.00-12.30	0.51 ± 1.34	1.08 ± 1.46	.286	0.65 ± 1.41	0.73 ± 1.15	.868
12.30-14.00	−0.43 ± 1.44	0.39 ± 1.45	.141	−0.12 ± 1.43	−0.12 ± 1.17	.988
14.00-16.00	−0.58 ± 0.73	0.71 ± 1.47	**.006**	−0.38 ± 0.78	0.49 ± 1.62	.076
16.00-19.30	0.27 ± 0.89	0.77 ± 1.58	.327	0.3 ± 0.78	0.56 ± 1.72	.599
19.30-21.00	0.55 ± 1.01	0.34 ± 1.6	.694	0.48 ± 1.07	0.15 ± 1.49	.504
21.00-23.00	0.35 ± 0.86	0.12 ± 1.47	.632	0.14 ± 0.97	0.18 ± 1.43	.918
07.00-23.00	0.15 ± 0.91	1.09 ± 1.69	.066	0.37 ± 1.22	0.69 ± 1.44	.525
07.00-14.00	0.06 ± 0.96	0.94 ± 1.79	.107	0.41 ± 1.55	0.44 ± 1.26	.958
12.30-16.00	−0.59 ± 0.89	0.72 ± 1.4	**.005**	−0.3 ± 1.01	0.32 ± 1.4	.181
14.00-23.00	0.18 ± 0.87	0.73 ± 1.61	.26	0.2 ± 0.82	0.54 ± 1.69	.494

Continuous data were presented as mean ± SD; *P* value was estimated through Student's *t*-test.
Significant differences are highlighted in bold.
AUC, area under the curve; HADS, Hospital Anxiety and Depression Scale; PSQI, Pittsburgh Sleep Quality Index.

or over exposure to GRT associated with factors impacting quality of life such as anxiety, depression, and sleep quality. Notably, salivary samples were collected at home and not in a hospital setting that may alter glucocorticoid rhythmicity in both patients and controls. Our findings confirm that current therapies may grant sufficient glucocorticoid levels, although there is no GRT able to perfectly mimic the physiologic glucocorticoid rhythmicity. In these regards, we also described a large inter-individual variability in salivary glucocorticoid levels at different time points, which is probably due to different GRT schemes and pharmacokinetics. In fact, salivary glucocorticoid in GRTs differed from controls at most of the time points and AUCs. Moreover, in accordance with other studies, the different GRT schemes we tested did not always result in similar glucocorticoid exposures.[21,22] Our study demonstrates that DRHC-od is superior when compared with other GRT schemes. Once-a-day DRHC provides the best glucocorticoid daily profile and is the most similar to control subjects. Nevertheless, DRHC-od is associated with higher glucocorticoid exposure in the morning and reduced exposure in the early afternoon compared with the physiologic rhythmicity.

Table 5. Linear and logistic regression for questionnaires prediction.

Dependent		Variables in the equation	Pearson's R^2	Coefficient	*P*
AddiQoL	Overall score	ZS-16:00	.194	−4.53	**.012**
		ZS-AUC$_{14:00\rightarrow16:00}$	.207	−4.77	**.009**
	Fatigue	ZS-16:00	.262	−2.18	**.003**
		ZS-AUC$_{14:00\rightarrow16:00}$	.28	−2.3	**.002**
	Symptoms	ZS-23:00	.128	1.06	**.044**
	Emotions	ZS-16:00	.182	−1.25	**.015**
		ZS-AUC$_{14:00\rightarrow16:00}$	.124	−1.05	**.048**
HADS	Overall score	ZS-16:00	.246	2.62	**.007**
		ZS-AUC$_{07:00\rightarrow07:30}$	.122	2.24	**.038**
		ZS-AUC$_{14:00\rightarrow16:00}$	.176	2.45	**.015**
	Anxiety	ZS-16:00	.217	1.32	**.012**
		ZS-AUC$_{07:00\rightarrow07:30}$	.171	1.27	**.029**
		Ongoing GRT scheme duration	.159	−2.35	**.036**
		ZS-AUC$_{14:00\rightarrow16:00}$	.284	1.03	**.015**
		Ongoing GRT scheme duration		−2.08	
	Depression	ZS-16:00	.216	1.29	**.013**
		ZS-AUC$_{14:00\rightarrow16:00}$	.204	1.28	**.016**
PSQI	Overall score	ZS-14:00	.215	1.39	**.013**
		ZS-AUC$_{14:00\rightarrow16:00}$	.265	1.15	**.005**
		ZS-AUC$_{12:30\rightarrow16:00}$	.259	1.18	**.006**
Dependent		Variables in the equation	Odds ratio	95% confidence of interval	*P*
Abnormal PSQI		ZS-AUC$_{14:00\rightarrow16:00}$	2.742	1.17-6.45	**.021**
		ZS-AUC$_{12:30\rightarrow16:00}$	2.428	1.14-5.16	**.021**

Significant differences are highlighted in bold.
AddiQoL, Adrenal insufficiency-specific Quality of Life; AUC, area under the curve; GRT, glucocorticoid replacement therapy; HADS, Hospital Anxiety and Depression Scale; PSQI, Pittsburgh Sleep Quality Index; ZS, *Z*-score.

Table 6. *Z*-score-AUC$_{14:00\rightarrow16:00}$ and AddiQol questionnaire.

AddiQoL	Z-score-AUC$_{14:00\rightarrow16:00}$		*P*
	<.619 (*n* = 24)	≥.619 (*n* = 9)	
Overall Score	89.4 ± 11.9	73.8 ± 12.1	**.002**
Fatigue	22 ± 5.3	15.7 ± 4.2	**.003**
Symptoms	28.3 ± 3.6	25.1 ± 4.5	**.047**
Emotions	24.5 ± 3.3	20.9 ± 4.3	**.015**

Continuous data were presented as mean ± SD; *P* value was estimated through Student's *t*-test.
Significant differences are highlighted in bold.
AUC, area under the curve; AddiQoL, Adrenal insufficiency-specific Quality of Life.

In accordance with a recent study, patients showed reduced salivary glucocorticoid levels at awakening compared with controls.[34] Interestingly, subjects taking CA showed higher glucocorticoid levels before drug intake compared with other patients. We cannot provide an exhaustive explanation with our data for this specific finding. However, since there is a majority of secondary AI patients in the CA group compared with other AI groups, we may speculate that it may be caused by a minimal residual glucocorticoid secretion as described by Vulto et al., or a different 11β-hydroxysteroid dehydrogenase type 1 metabolism as described by Sherlock et al.[35,36] In these regards, persistence of excess glucocorticoid exposure throughout the night could be explored by measuring nighttime glucocorticoid levels and possibly explain the higher glucocorticoid values obtained at awakening time in the CA.

One or two GRT administrations do not seem to affect afternoon and evening *Z*-score values in HC and CA patients. On the contrary, due to its peculiar pharmacokinetics, splitting DRHC in 2 administrations leads to higher glucocorticoid exposure throughout the day compared to DRHC-od and other GRTs.

Higher glucocorticoid levels in the afternoon and evening were independent risk factors for worse sleep quality, quality of life, increased anxiety, and depressive symptoms, regardless of type of GRT, overall GRT duration, HC-equivalent dose, and aetiology or duration of AI. In details, these psychological disturbances are mostly present in patients with salivary glucocorticoid *Z*-score values ≥ +.619 from 14:00 to 16:00. These findings are consistent with previous studies on cortisol rhythmicity, allowing to hypothesize that an unbalanced GRT may worsen several aspects of sleep and life quality.[23,24] The mean dose intake in our cohort exceeds the maximum dose recommended by a previous study.[37] Interestingly, in our study, the GRT daily dose seems not to be associated with psychological disturbances. Rarely, low *Z*-score night levels (23:00) were associated with worse scores on the AddiQoL questionnaire. This suggests that, in selected patients, low night glucocorticoid levels may have an impact on quality of life. These findings have to be confirmed in studies on larger cohorts and focusing on nighttime kinetics.

According to our results, lower duration of current GRT is associated with increased risk of anxiety, probably reflecting the need to watch carefully patients undergoing GRT adjustments. Moreover, increased risk of anxiety is also associated with higher glucocorticoid exposure after first GRT administration, probably due to non-physiological and too steep increase in glucocorticoid levels, but it should be verified in targeted studies.

This is the first study employing *Z*-score measures of salivary glucocorticoids as dimensionless indicators allowing the comparison of several GRTs. *Z*-score seems a useful surrogate for glucocorticoid exposure estimation, with the ability to predict

Table 7. *Z*-score-AUC$_{14:00\rightarrow16:00}$ and PSQI and HADS questionnaires.

Z-score-AUC$_{14:00\rightarrow16:00}$	Normal PSQI ($n = 13$)	Altered PSQI ($n = 16$)	*P*	Normal HADS ($n = 14$)	Altered HADS ($n = 15$)	*P*
≥ .619	0 (.0%)	8 (50.0%)	**.003**	1 (7.1%)	6 (40.0%)	**.039**

Categorical data were presented as proportion (%); *P* value was estimated through Pearson's χ^2.
Significant differences are highlighted in bold.
AUC, area under the curve; HADS, Hospital Anxiety and Depression Scale; PSQI, Pittsburgh Sleep Quality Index.

life quality aspects. However, the *Z*-score approach needs to be confirmed by studies in larger populations with interventional longitudinal design. Moreover, this technique may be useful for the management of other types of AI such as glucocorticoid-induced AI. If confirmed, given the limitations shown by the available GRTs, the decision-making process for the possible modification of GRT dose or scheme should consider high salivary glucocorticoid *Z*-score in early afternoon in association with poor life quality and the overall clinical presentation.

The limitations of this study are due to its cross-sectional design and a small sample of patients, which is expected in rare entities such as adrenal insufficiency. Nevertheless, our study cohort was comparable in size with previous studies.[19-22] The strengths of our study rely on the thorough characterization of a highly selected population, the highly sensitive and specific quantification of SalF and SalE by LC-MS/MS technology, and a glucocorticoid drug contamination-free comparison system.

Conclusions

SalF and SalE measurements may be useful in the assessment of GRT in adrenal insufficiency. Their measurements may establish clearer differences among GRT schemes. There is no GRT that mimics and restores physiological glucocorticoid rhythm. However, DRHC-od has been shown to provide the best diurnal glucocorticoid profile. *Z*-score of SalF and SalE may be a suitable tool for comparison of several GRTs with different compounds. High glucocorticoid exposure in the second part of the day seems to associate with poor quality of life, poor sleep quality, and depressive symptoms. Further confirmations are needed through prospective studies in wider population.

Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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Authors' contributions

Lorenzo Tucci (Conceptualization [lead], Data curation [lead], Formal analysis [lead], Investigation [lead], Methodology [equal], Project administration [equal], Software [equal], Writing—original draft [lead]), Flaminia Fanelli (Conceptualization [supporting], Data curation [equal], Funding acquisition [equal], Methodology [equal], Resources [equal], Supervision [equal], Validation [equal], Writing—review & editing [equal]), Ilaria Improta (Investigation [supporting]), Valentina Bissi (Investigation [supporting]), Claudia Lena (Investigation [supporting]), Greta Galante (Investigation [supporting]), Marco Mezzullo (Investigation [supporting]), Matteo Magagnoli (Investigation [supporting]), Anna Bianca Lalumera (Investigation [supporting]), Giacomo Colombin (Investigation [supporting]), Kimberly Coscia (Investigation [supporting]), Laura Rotolo (Investigation [supporting]), Valentina Vicennati (Conceptualization [supporting], Investigation [supporting], Methodology [supporting], Writing—review & editing [equal]), Uberto Pagotto (Funding acquisition [equal], Supervision [equal], Writing—review & editing [equal]), and Guido Di Dalmazi (Conceptualization [lead], Data curation [equal], Formal analysis [equal], Investigation [equal], Methodology [equal], Project administration [equal], Resources [equal], Software [equal], Supervision [equal], Validation [equal], Writing—review & editing [lead])

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