RESEARCH

Confounding effects of liquorice, hydrocortisone, and blood contamination on salivary cortisol but not cortisone

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Abstract

Objective: To determine the effects of liquorice consumption, topical hydrocortisone, and blood contamination on salivary cortisol and cortisone concentrations.
Design and methods: Thirty healthy volunteers were randomized to a low, medium, or high dose of liquorice. Late-night saliva samples were collected using a Salivette® collection device at baseline, during 1 week of daily liquorice consumption, and during 4 weeks' washout. Saliva sampling was also performed before and after the application of topical hydrocortisone on the skin. Furthermore, in a subgroup (n = 16), saliva and venous blood were collected from each individual and mixed to achieve graded blood contamination in saliva. Salivary cortisol and cortisone were analyzed with liquid chromatography-tandem mass spectrometry.
Results: Significant increases in salivary cortisol concentrations were observed during medium- (+49%) and high-dose (+97%) liquorice intake, which returned to baseline 4 days after liquorice withdrawal. Topical hydrocortisone on fingers holding the collection swab increased salivary cortisol concentrations >1000-fold with concomitant pronounced elevation of the cortisol:cortisone ratio. Salivary cortisol increased significantly after contamination with blood ≥0.5%. Visual examination could safely detect these samples. Salivary cortisone concentrations were unaffected by liquorice consumption and blood contamination, and only marginally affected by topical hydrocortisone.
Conclusion: Liquorice, topical hydrocortisone, and blood contamination may all cause elevated salivary cortisol concentrations. Improved sampling instructions and visual examination of the sample may minimize these risks. Salivary cortisone is essentially unaffected by the different preanalytical confounders and may be used as a first-line screening test for Cushing's syndrome.

Key Words
- Cushing's syndrome
- salivary cortisol
- salivary cortisone
- liquorice
- sample contamination

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Introduction

Timely diagnosis of endogenous Cushing's syndrome (CS) is important to reduce morbidity and mortality (1, 2, 3). Late-night salivary cortisol (LNSC) is an important screening instrument for CS with high sensitivity and specificity (4, 5, 6). Cortisol analyses using liquid chromatography tandem mass spectrometry (LC-MS/MS) have eliminated the problems of cross-reactivity with exogenous steroids and cortisol metabolites often found with immunoassays (7, 8, 9, 10). However, salivary cortisol may be falsely elevated due to preanalytical errors

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including liquorice consumption and contamination with dermal hydrocortisone or blood in the saliva sample (11, 12, 13). Salivary cortisone might be less sensitive to these problems and has been suggested to be superior to salivary cortisol as a screening tool for CS (14, 15).

The 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme converts biologically active cortisol into inactive cortisone, e.g. in the colon, the renal collecting ducts, and the salivary glands (16, 17, 18). Glycyrrhetinic acid, a metabolite of the liquorice compound glycyrrhizic acid (GA), inhibits 11β-HSD2 (19, 20). Thus, liquorice intake may potentially increase cortisol concentrations and the cortisol:cortisone ratio in saliva. However, dose–effect relationships, time-course of effect, and possible effect on salivary cortisone concentrations have not been evaluated (7, 13).

Furthermore, although contamination with topical hydrocortisone has been repeatedly reported as a known preanalytical error leading to increased salivary cortisol concentrations, there is a shortage of published data on the effects on both salivary cortisol and cortisone (21, 22).

A third confounder for salivary cortisol is blood contamination of the sample from rifts in the oral mucosa. However, previous studies have not evaluated whether salivary cortisone is influenced (11, 23, 24).

In order to establish the importance of potential preanalytical errors for salivary cortisol and cortisone, we have analyzed: (i) the GA dose needed to significantly increase LNSC or cortisone concentrations and the washout period required for these changes to normalize; (ii) the effect of topical hydrocortisone cream use before saliva sampling on salivary cortisol and/or cortisone concentrations; and (iii) what blood concentration in saliva might increase cortisol and/or cortisone concentrations and how these samples can be detected.

Materials and methods

Study population

Participants were recruited through flyers at Umeå University Hospital and Umeå University grounds as well as through social media. Inclusion criteria were age 18–65 years and total abstinence from liquorice for 4 weeks prior to the study start. Exclusion criteria were known pituitary and/or adrenal gland disease; current glucocorticoid therapy (including inhalations, nasal sprays, skin creams, etc.); hypertension or use of antihypertensive drugs; smoking or other tobacco use; problems with the oral mucosa or saliva; current pregnancy; abnormal circadian rhythm (not sleeping at night between 00:30 and 05:00 h). The study was approved by the local ethics committee in Umeå (Dnr 2018-162-31M). Written informed consent was obtained from all participants prior to study start.

Saliva sampling

Participants were instructed to avoid strenuous exercise, eating, and brushing teeth 1 h prior to saliva sampling. Drinking was allowed up to 30 min before sampling, and the participants were instructed to rinse their mouth with water 10 min prior to sampling. Saliva was collected with a Salivette® Cortisol swab collection device (Sarstedt, Nümbrecht, Germany) by tipping the opened Salivette® tube to allow the synthetic swab to slide into the mouth before either gently chewing the swab for 60 s or keeping it under the tongue for 90 s and then spitting it back into the tube without touching the swab with the fingers.

Liquorice study

Thirty volunteers (22–55 years of age, 15 females) were recruited for the 6-week liquorice study. Height and body weight were measured at the first visit to the clinical research center and blood pressure was measured at all visits. Venous blood samples were taken by antecubital venipuncture for analysis of plasma sodium, potassium, and creatinine on days 1, 14, and 21 of the study (Fig. 1). During the baseline week, participants were instructed to collect saliva samples at home for five consecutive mornings (06:00–08:00 h) and nights (22:00–23:00 h) and store the samples in their home freezers until the next visit to the clinical research center (Fig. 1). Seven days after the first visit, participants returned for randomization to a daily liquorice dose corresponding to GA 1.5, 3.0, or 6.0 mg/kg (low, medium, and high doses, respectively). The liquorice used in the study consisted of pure liquorice extract without any additives (Tronchetti 1886, Calabria, Italy). The GA content in the liquorice was analyzed by Analiticals Controls S.R.L (San Marco Argentano, Calabria Italy) with standard high-performance LC established by the Association of Official Analytical Chemists (25). The GA content in the liquorice was 1.95 g per 100 g liquorice. The liquorice was weighed with a 0.1 g precision scale and each participant received seven zip-lock plastic bags containing their respective daily doses. During the 'liquorice' week, participants were instructed to consume one bag of liquorice daily for 7 days, between 18:00 and 21:00 h, with fasting 90 min before the

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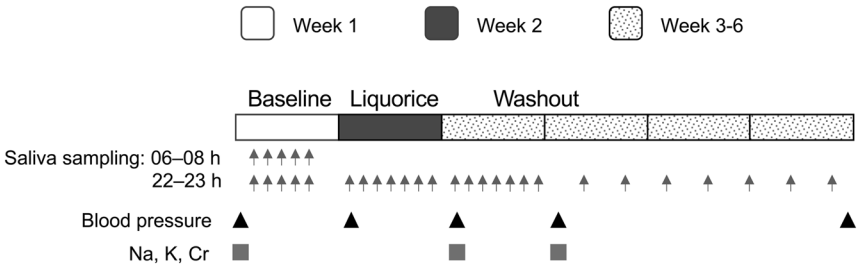

Figure 1
Schematic view of the 6-week study protocol for all 3 GA dose groups 1.5, 3.0, and 6.0 mg/kg body weight. Cr, plasma creatinine; K, plasma potassium; Na, plasma sodium.

liquorice intake and to perform saliva sampling every night as previously described. This was followed by a 4-week washout period during which the participants refrained from all types of liquorice consumption. During 'washout', saliva was collected every night during the first week and every third night during weeks 2–4 after stopping liquorice intake (Fig. 1). Baseline characteristics of the participants are presented in Table 1.

Topical hydrocortisone cream study

Participants in the hydrocortisone study ($n = 30$) first collected a saliva sample, between 13:00 and 17:00 h, with the specific instruction to pick the synthetic swab up with their dominant thumb, index, and middle fingers and place it in their mouth for 60–90 s before restoring the swab to the Salivette® Cortisol tubes using the same fingers. Thereafter, the participants received approximately 1 cm hydrocortisone cream 1% (Mildison Lipid, Karo Pharma, Stockholm, Sweden) and were instructed to use their dominant index and middle fingers to apply the cream on the back of their non-dominant hand before another saliva sample was collected 10 min after the first sample in exactly the same way.

Another five participants performed the same procedure as mentioned earlier with the inclusion of a white Klinion® Ultra Comfort nitrile examination glove (Medeco BV, Oud-Beijerland, Netherlands) put on the dominant hand after applying the hydrocortisone cream.

Blood contamination study

Sixteen volunteers (23–50 years of age, 8 females) were included in the blood contamination study, 12 of whom had previously participated in the liquorice and hydrocortisone studies. To mimic contamination of the patients' own blood from a mucosal rift at high and low cortisol concentrations saliva samples (10 mL) were collected between 07:00 and 09:00 h by passive drooling using Saliva Collection Aid® (Salimetrics, State College, PA, USA) and 50 mL screw cap tubes (Sarstedt). Immediately after this, blood was collected by antecubital venipuncture in BD Vacutainer® K2-EDTA tubes (Becton Dickinson,

Table 1 Baseline characteristics of the liquorice groups based on GA dose received.

		Glycyrrhizic acid dose			
Characteristics	Total ($N = 26$)	1.5 mg/kg ($n = 9$)	3.0 mg/kg ($n = 9$)	6.0 mg/kg ($n = 8$)	*P*-value
Female	12 (46.2%)	4 (44.4%)	4 (44.4%)	4 (50%)	NS
Age (years)	37 ± 8	38 ± 9	34 ± 5	40 ± 10	NS
Height (cm)	171.5 ± 10.5	173.1 ± 8.1	172.3 ± 12.3	169.0 ± 11.8	NS
Weight (kg)	75.6 ± 12.7	78.0 ± 11.8	70.3 ± 12.5	78.9 ± 13.4	NS
BMI (kg/m^2)	25.6 ± 3.5	26.0 ± 3.8	23.5 ± 1.5	27.6 ± 3.7	0.048[a]
SBP (mmHg)	114 ± 11	113 ± 8	113 ± 11	118 ± 14	NS
DBP (mmHg)	74 ± 6	73 ± 5	71 ± 5	78 ± 7	NS
Plasma sodium (mmol/L)	140 ± 2	140 ± 1	140 ± 2	140 ± 2	NS
Plasma potassium (mmol/L)	3.9 ± 0.3	4.0 ± 0.3	3.9 ± 0.3	3.9 ± 0.2	NS
Plasma creatinine (µmol/L)	75 ± 12	72 ± 10	71 ± 11	84 ± 13	NS
LNSC (nmol/L)	0.72 (0.50–1.20)	0.75 (0.51–1.20)	0.67 (0.47–1.20)	0.74 (0.50–1.16)	NS
LNSa-cortisone (nmol/L)	4.45 (3.19–6.27)	4.43 (2.81–5.63)	4.66 (3.41–6.57)	4.06 (3.19–6.50)	NS
Cortisol/cortisone ratio	0.16 (0.13–0.22)	0.17 (0.14–0.23)	0.14 (0.12–0.18)	0.16 (0.15–0.21)	0.013[b]

Data are presented as *n* (%), mean ± S.D., or median (interquartile range).
[a]Difference between 3.0 and 6.0 mg/kg.
[b]Difference between 1.5 and 3.0 mg/kg.
DBP, diastolic blood pressure; LNSa-cortisone, late-night salivary cortisone; LNSC, late-night salivary cortisol; NS, not statistically significant; SBP, systolic blood pressure.

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Franklin Lakes, NJ, USA). The participants then performed a dexamethasone suppression test (DST), i.e. instructed to take a 1-mg dexamethasone tablet (Abcur, Helsingborg, Sweden) at home between 22:00 and 23:00 h and return the following morning between 07:00 and 09:00 h to the clinical research center for repeated saliva and blood sample collection as mentioned earlier. Saliva samples were vortexed for 10 s and EDTA tubes with blood were carefully inverted ten times. Saliva and blood from the same participant and timepoint were mixed in six 5-mL tubes 75 × 13 mm (Sarstedt) to a final volume of 1 mL in each with a blood content of 0, 1, 5, 10, 20, and 40 µL (0, 0.1, 0.5, 1, 2, and 4%, respectively). A Salivette® Cortisol swab was placed in each of the tubes for 2–3 min to absorb the blood–saliva mixture. The swabs were then carefully transferred into Salivette® Cortisol tubes and stored in a freezer (–20°C) for later analysis of cortisol and cortisone by LC-MS/MS. After analysis, the samples were stored in a freezer (–80°C) until visual grading after thawing and vortexing. Three independent observers visually evaluated each saliva sample without swab using a 7-point grading scale (1 = saliva appears clear, no visible color; 2 = saliva has a hint of color that is barely visible; 3 = saliva has a visible pink tint; 4 = saliva is distinctly pink colored; 5 = saliva is light red colored; 6 = saliva is distinctly red colored; 7 = saliva is dark red colored). An interobserver analysis of the visual inspection showed that at least two out of three independent observers graded the sample with the same score in 100% of all samples. When dividing the 7-point grading into a binary grading (grades 1–2 = no blood contamination and grades 3–7 = contaminated with blood), the score was the same for all three independent observers in 99% of all saliva samples. Recovery of salivary cortisol in blood-contaminated saliva was compared with calculated salivary cortisol based on plasma cortisol concentration and erythrocyte volume fraction (plasma cortisol concentration × (1 – erythrocyte volume fraction) × blood contamination concentration + baseline salivary cortisol concentration).

Exclusions and missing samples

Of the 30 volunteers initially recruited for the liquorice study, 4 were excluded due to elevated blood pressure ($n = 1$), inability to consume the liquorice ($n = 1$), or consent withdrawal ($n = 2$). Of the total 806 planned saliva samples from the remaining 26 participants, 29 samples from 14 participants were lacking due to missed saliva collection or collection >1 h outside of the instructed sampling time during baseline ($n = 12$), liquorice week ($n = 3$), washout week 1 ($n = 8$), and washout weeks 2–4 ($n = 6$). For two participants, one of the five baseline samples was excluded due to a cortisol:cortisone ratio ≥1 (5, 7).

In the blood contamination study, saliva samples with inadequate cortisol suppression (>0.79 nmol/L) after DST were excluded ($n = 3$) as the aim was to study the effects of blood contamination at low salivary cortisol concentrations (Supplementary Table 1) (5).

Analytical methods

The Salivette® Cortisol tubes with saliva in the swabs were thawed and centrifuged at 3000 ***g*** for 10 min, after which the synthetic swabs were discarded. The samples contaminated with topical hydrocortisone were found to have very high cortisol levels in initial analyses and were therefore diluted 1:100 with PBS prior to analysis to avoid cortisol overloading. Saliva concentrations of cortisol and cortisone were determined by LC-MS/MS after sample preparation using supported liquid extraction, as described previously (5, 10). In brief, 130 µL of each sample was mixed with 130 µL of a water solution containing the deuterated internal standards cortisol-D_4 (Cambridge Isotope Laboratories, Tewksbury, MA, USA) and cortisone-D_7 705586 (Sigma-Aldrich) and were then purified by supported liquid extraction on Isolute SLE+ 200 µL Array wells (Biotage, Uppsala, Sweden) and analyzed on a Nexera UHPLC system (Shimadzu, Kyoto, Japan) coupled to a QTRAP® 5500 MS/MS (SCIEX, Framingham, MA). This method has been validated to efficiently separate prednisolone and the two endogenous cortisol isomers 20α- and 20β-dihydrocortisone from the analytes. The interassay coefficient of variation (CV_A) for cortisol was 7 and 6% at control concentrations 1.4 and 50 nmol/L, respectively. The CV_A for cortisone was 7% for both control concentrations 4 and 110 nmol/L. The lower limit of quantification was originally defined as 0.25 nmol/L for cortisol and 0.20 nmol/L for cortisone. However, since a revalidation of total imprecision at a cortisol concentration of 0.27 nmol/L revealed a total CV_A of 12%, values below the limit of quantification for cortisol were also included in the analyses.

Blood pressure was recorded as the mean of the second and third of three measurements on the right arm in a sitting position after 5-min rest. Erythrocyte volume fraction was examined on Sysmex XN-9000 hematology analyzer (Sysmex, Kobe, Japan). Plasma concentrations of sodium, potassium, and creatinine were determined on Cobas® 8000 modular analyzer series (Roche Diagnostics) in the ISO 15189 accredited laboratory at Umeå University Hospital.

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Statistics

Between-group comparisons of the baseline characteristics of the three dose groups in the liquorice study were analyzed using Kruskal–Wallis *H*-test followed by *post hoc* Mann–Whitney *U*-test. For all analyses of exposure effects on salivary cortisol and cortisol:cortisone ratio, one-tailed statistical tests were used as the *a priori* hypotheses were exclusively increased after experimental exposures. For salivary cortisone, two-tailed statistical analyses were used.

The within-subject coefficient of variation (CV_I) and the between-subject coefficient of variation (CV_G) for salivary cortisol and cortisone were calculated based on the baseline samples from the 26 participants in the liquorice study. Differences in CV_I between salivary cortisol and salivary cortisone were tested using the Wilcoxon signed-rank test and differences in CV_G using the test by Feltz and Miller (26).

The effect of liquorice was tested using the Wilcoxon signed-rank test using the median of each individual's late-night baseline samples vs the median of the same individual's concentrations during the liquorice week for each day of washout separately. Furthermore, three sources of inherent variation exist in each individual's biochemical test, such as salivary cortisol, i.e. preanalytical confounders (e.g. liquorice intake), analytical imprecision, and within-subject biological variation. Hence, each LNSC and late-night salivary cortisone concentration in the time series during and after liquorice intake was compared with the individual's own reference change value (RCV). The RCV calculates an individual's range based on the analytical imprecision and the within-subject biological variation; thus, values exceeding this range are considered significantly altered by preanalytical confounders. RCV is calculated according to the formula $RCV = \sqrt{2} \times Z \times \sqrt{(CV_A^2 + CV^2)}$ where Z is the number of standard deviations corresponding to 95% probability of a unidirectional change (Z = 1.65 for salivary cortisol) or bidirectional change (Z = 1.96 for salivary cortisone) (27), CV_A is the interassay coefficient of variation for the analytical method (7% for both cortisol and cortisone), and CV is each individual's CV based on the baseline late-night salivary samples.

Effects of liquorice on blood pressure and plasma potassium were analyzed using the Friedman test followed by *post hoc* analyses with Wilcoxon signed-rank test.

The Wilcoxon signed-rank test was also used for analyzing the effects of topical hydrocortisone on salivary cortisol, cortisone, and cortisol:cortisone ratio.

The effect of blood contamination was tested using the Friedman test followed by *post hoc* analyses with Wilcoxon signed-rank test with Bonferroni correction. To assess whether clinically relevant blood contamination of the same aliquoted saliva sample changed the cortisol or cortisone concentration more than the inherent variability due to both analytical imprecision and within-subject variability, similarly to RCV, each sample was compared with the total change limit (TCL) for the analyte, calculated with the formula $TCL = \sqrt{((Z \times \sqrt{2} \times CV_A)^2 + (0.5 \times CV_B)^2)}$ (28). For the unsuppressed blood-contaminated samples, CV_B was calculated as the within-subject coefficient of variation from the 5 morning baseline samples from the 12 participants included in both the liquorice study and the blood contamination study and $CV_A = 7\%$ was used. For the blood-contaminated saliva samples collected after DST, CV_B was calculated from the five late-night baseline samples and CV_A for very low cortisol was used instead (12%).

All analyses were carried out using IBM SPSS statistical software package for Macintosh, version 26.0 (IBM Corp.), except the test by Feltz and Miller carried out using the R package cvequality (Version 0.1.3) (29). The statistical significance threshold was set to $P < 0.05$.

Results

Intra- and interindividual variability of salivary cortisol and cortisone

For unsuppressed morning salivary cortisol and cortisone, CV_I was 35 and 22% ($P < 0.001$), and CV_G was 49 and 26% ($P = 0.006$) at mean concentrations of 10.3 and 32.5 nmol/L, respectively. For LNSC and late-night salivary cortisone, CV_I was 49 and 37% ($P = 0.002$), and CV_G was 95 and 66% ($P = 0.238$) at mean concentrations of 0.96 and 5.29 nmol/L, respectively.

Liquorice

Median LNSC was significantly elevated during liquorice consumption in the medium- (+49%; $P = 0.004$) and high-dose groups (+97%; $P = 0.006$) compared to baseline (Fig. 2, Table 2). During washout, LNSC was still significantly higher than baseline on day 2 of washout in the medium-dose group and on day 3 of washout in the high-dose group. Late-night salivary cortisone was not significantly altered by any of the liquorice doses. The salivary cortisol:cortisone ratio showed similar changes to LNSC with a significant increase during liquorice consumption in the medium- and high-dose groups ($P = 0.004$ and

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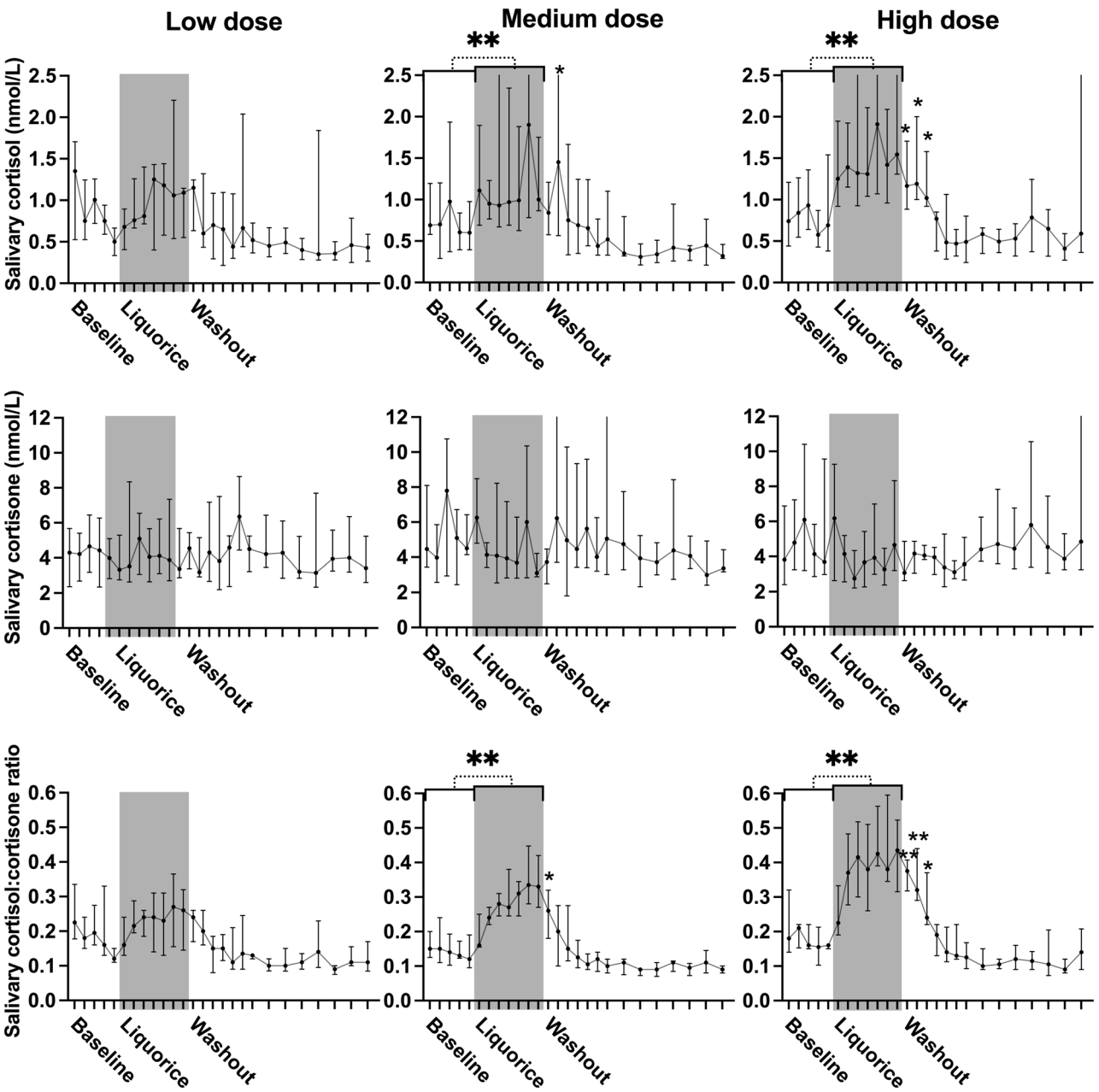

Figure 2
Late-night salivary cortisol, cortisone, and cortisol:cortisone ratio before, during, and after liquorice intake in the three GA dose groups 1.5 (n = 9), 3.0 (n = 9), and 6.0 (n = 8) mg/kg body weight. Data presented as medians with interquartile ranges. *P < 0.05 and **P < 0.01 compared to baseline using Wilcoxon signed-rank test.

P = 0.006, respectively) (Fig. 2, Table 2). Separate analyses of males and females showed significant increases of LNSC in both sexes during liquorice consumption in both the medium- (P = 0.021 and P = 0.034, respectively) and high-dose groups (P = 0.034 and P = 0.034, respectively) (data not shown). To evaluate the magnitude of the liquorice effect in relation to analytical and individual variability, we compared the liquorice-induced changes with RCV. LNSC exceeded RCV for the low-, medium-, and high-dose groups in 11, 39, and 44% of the samples collected during the liquorice week, returning to levels within RCV at day 6 of washout (Supplementary Fig. 1, see section on supplementary materials given at the end of this article).

Blood pressure and plasma potassium concentrations were not significantly affected by 1 week of low- or medium-dose liquorice consumption. However, after 1

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Table 2 Salivary cortisol and cortisone concentrations, and cortisol:cortisone ratios during baseline, liquorice intake, and washout days 1–5 for the three GA doses.

			Washout days				
GA dose	Baseline	Liquorice intake	1	2	3	4	5
1.5 mg/kg (n = 9)							
LNSC (nmol/L)	0.75 (0.51–1.20)	0.91 (0.65–1.34)	0.93 (0.59–1.28)	0.59 (0.40–0.72)	0.70 (0.21–1.12)	0.65 (0.21–0.73)	0.40 (0.27–0.68)
LNSa-cortisone (nmol/L)	4.43 (2.81–5.63)	3.83 (2.98–5.87)	4.54 (3.96–5.35)	3.18 (2.95–3.69)	4.31 (3.02–6.20)	3.81 (2.24–5.00)	4.59 (2.91–5.18)
Cortisol:cortisone ratio	0.17 (0.14–0.23)	0.23 (0.15–0.29)	0.24 (0.22–0.25)	0.20 (0.19–0.26)	0.15 (0.09–0.18)	0.15 (0.13–0.17)	0.11 (0.09–0.19)
3.0 mg/kg (n = 9)							
LNSC (nmol/L)	0.67 (0.47–1.20)	1.00[a] (0.77–1,80)	0.84 (0.71–1.20)	1.45[b] (0.60–1.87)	0.75 (0.41–1.25)	0.69 (0.39–1.17)	0.66 (0.48–1.14)
LNSa-cortisone (nmol/L)	4.66 (3.41–6.57)	4.11 (3.02–6.25)	3.72 (2.52–4.42)	6.22 (4.51–12.6)	4.97 (2.10–7.58)	4.47 (3.47–8.48)	5.63 (4.01–9.28)
Cortisol:cortisone ratio	0.14 (0.12–0.18)	0.28[a] (0.22–0.33)	0.26[b] (0.19–0.29)	0.20 (0.10–0.25)	0.15 (0.13–0.27)	0.13 (0.10–0.16)	0.11 (0.09–0.13)
6.0 mg/kg (n = 8)							
LNSC (nmol/L)	0.74 (0.50–1.16)	1.46[a] (1.06–2.13)	1.17[b] (0.90–1.53)	1.19[b] (1.00–2.00)	1.02[b] (0.92–1.58)	0.77 (0.38–0.85)	0.49 (0.30–0.95)
LNSa-cortisone (nmol/L)	4.06 (3.19–6.50)	4.00 (2.58–5.73)	3.07 (2.71–4.68)	4.16 (3.05–4.86)	4.05 (3.82–4.63)	3.95 (2.98–4.52)	3.38 (2.48–5.28)
Cortisol:cortisone ratio	0.16 (0.15–0.21)	0.38[a] (0.30–0.49)	0.38[a] (0.33–0.41)	0.32[a] (0.29–0.44)	0.24[b] (0.22–0.37)	0.19 (0.13–0.22)	0.14 (0.12–0.21)

Data are presented as median (interquartile range).
[a]P < 0.01 compared to baseline (Wilcoxon signed-rank test).
[b]P < 0.05 compared to baseline (Wilcoxon signed-rank test).
GA, glycyrrhizic acid; LNSa-cortisone, late-night salivary cortisone; LNSC, late-night salivary cortisol.

week of high-dose liquorice consumption, mean systolic blood pressure was significantly increased (+9.2 mmHg; P = 0.030) and mean plasma potassium was significantly decreased (–0.5 mmol/L; P = 0.003). Diastolic blood pressure was not significantly altered (Fig. 3). Hypokalemia (<3.5 mmol/L) was found in six of the eight participants in the high-dose group.

Topical hydrocortisone

Application of topical hydrocortisone cream on fingers a few minutes prior to holding the Salivette® swab resulted in a >1000-fold increase of median salivary cortisol concentrations (P < 0.001) and a pronounced increase in the cortisol:cortisone ratio from <1 to ≥30 among all participants (P < 0.001) (Fig. 4). A small, but significant, increase was also seen for salivary cortisone (+32%; P=0.001). When using a nitrile glove on the hydrocortisone cream-contaminated hand, no significant changes were seen in salivary cortisol or cortisone, or cortisol:cortisone ratio (data not shown).

Blood contamination

Blood used to spike saliva samples had a median plasma cortisol concentration of 342 nmol/L (interquartile range 227–438 nmol/L) before DST and 21 nmol/L (interquartile range 16–24 nmol/L) after DST. Compared with expected salivary cortisol in blood-spiked samples, the mean recovery of cortisol in 4% blood-contaminated saliva samples was 107% before DST and 115% after DST (Supplementary Fig. 2). In unsuppressed morning samples, blood contamination of 0.5% significantly increased median salivary cortisol (+17%; adjusted P = 0.003) compared to uncontaminated saliva (Fig. 5). Significantly increased salivary cortisol was also found in samples collected after DST at 0.5% blood contamination (+122%; adjusted P = 0.013) compared to uncontaminated saliva. The cortisol:cortisone ratio was also significantly increased at 0.5% blood contamination in both unsuppressed (adjusted P = 0.003) and suppressed samples (adjusted P = 0.015). To evaluate the magnitude of the effect of blood contamination in relation to analytical and individual variability, we also compared the alterations using TCL. Mean salivary cortisol concentration exceeded the TCL at 1% blood contamination for unsuppressed morning samples and at 0.5% blood contamination for suppressed (with DST) morning samples. Salivary cortisone concentrations were not significantly changed by blood contamination up to the maximum tested concentration of 4% blood (Fig. 5). Accordingly, the variability of salivary

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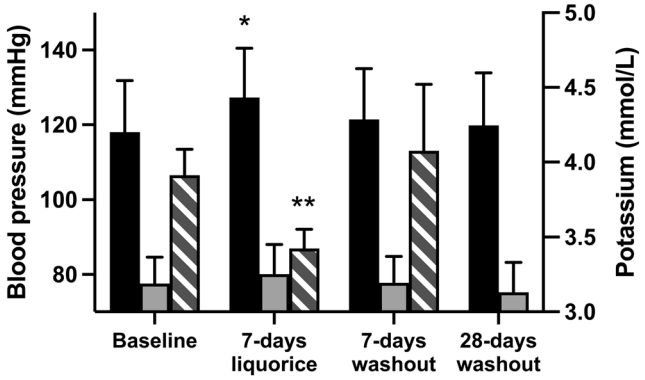

Figure 3
Systolic and diastolic blood pressures, and plasma potassium concentrations in the high-dose group (GA 6.0 mg/kg, n = 8) at baseline, after 7 days of liquorice consumption, and 7 and 28 days after stopping liquorice intake. Data presented as means + S.D. *P < 0.05 and **P < 0.01 compared to baseline using Wilcoxon signed-rank test. Black bars, systolic blood pressure; grey bars, diastolic blood pressure; hatched bars, plasma potassium.

cortisol but not salivary cortisone increased with increasing blood concentrations. Visual inspection of the samples using the binary grading 'blood contaminated' or 'not blood contaminated' detected samples with ≥0.5% blood contamination with 98% sensitivity and 100% specificity.

Discussion

The main finding in this study is that salivary cortisone concentrations were unaffected by liquorice consumption and blood contamination, and only marginally affected by topical hydrocortisone. In contrast, salivary cortisol was increased significantly by liquorice consumption corresponding to GA ≥3.0 mg/kg body weight, lasting several days after cessation of liquorice intake. We also found profoundly increased salivary cortisol after handling topical hydrocortisone close to saliva sampling and a significant increase with ≥0.5% blood contamination of the saliva.

Salivary cortisol was significantly increased in both males and females after liquorice consumption in our study. This is in contrast to the only (to our knowledge) previous study on the effect of liquorice on salivary cortisol, in which consumption of liquorice 100 g/day for a week (comparable to our medium dose group of GA 3.0 mg/kg) significantly increased salivary cortisol concentrations only in female participants (13). This difference with lack of significant effect in males in the study by Al-Dujaili and colleagues (13) might be explained by a lower GA dose per kg bodyweight due to the fixed liquorice dose for all participants compared to dosing of liquorice per kg body weight in our study.

The amount of GA in various types of confectionary liquorice varies substantially between manufacturers and even between different batches from the same manufacturer, in one study from 0.26 to 7.9 mg GA per gram liquorice from different confectionery manufacturers (30), and in a more recent study from <0.01 to 23.2 mg GA with a mean of 2.0 mg per gram confectionery liquorice (31). Using an approximation of 2 mg GA per gram confectionery liquorice, our medium dose would correspond to 100 g of confectionery liquorice for a person weighing 65–70 kg.

Previous studies have not investigated the duration of the effect after stopping liquorice intake (13, 32, 33, 34, 35). Thus, a novel finding in our study is that salivary cortisol concentrations remained significantly elevated up to 3 days after stopping liquorice consumption. In a clinical setting, the maximum tolerated confounding effect of a preanalytical error is related to other sources of variability in the analysis, i.e. analytical variability and biological variability in RCVs. Salivary cortisol still exceeded the RCV up to 5 days after stopping liquorice intake. As GA content varies in liquorice confectionery, we suggest that sampling instructions for LNSC sample collection should include total refrainment from all liquorice consumption for 1 week before sampling.

One week of high-dose liquorice intake significantly increased systolic blood pressure and decreased potassium

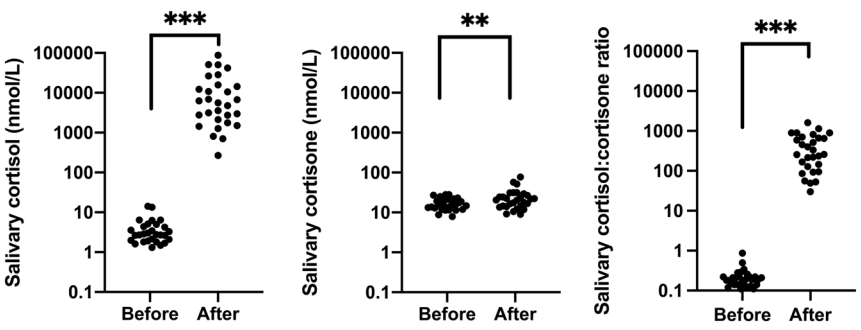

Figure 4
Salivary cortisol, cortisone, and cortisol:cortisone ratio, collected in the afternoon, before and after application of topical hydrocortisone cream on fingers handling the Salivette® swab. $N = 29$. **P < 0.01 and ***P < 0.001 using Wilcoxon signed-rank test. Note logarithmic scale.

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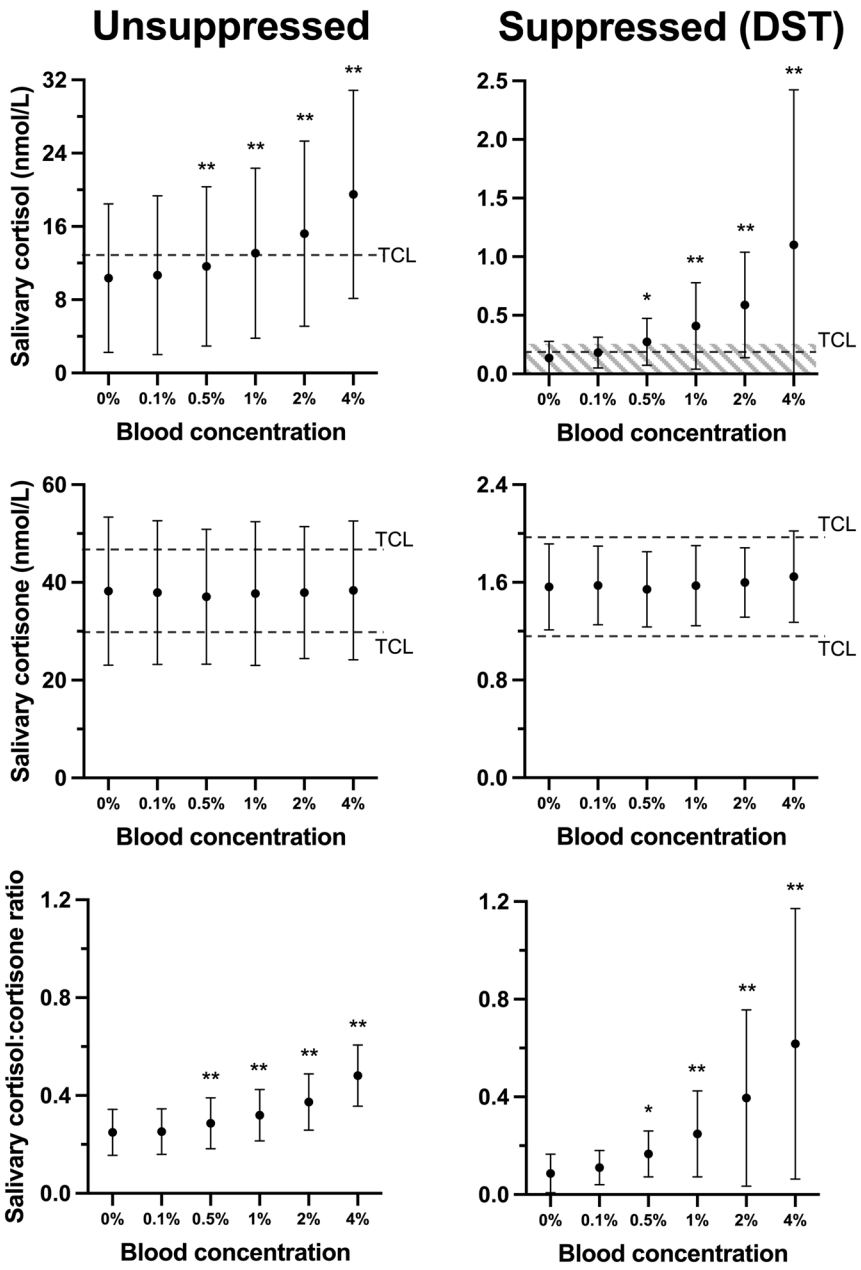

Figure 5
Salivary cortisol, cortisone, and cortisol:cortisone ratio in unsuppressed (n = 16) and suppressed (DST 1 mg, n = 13) morning samples with different concentrations of blood contamination. Gray striped area marks lower limit of quantification (upper right panel). Horizontal dashed lines indicate the total change limit (TCL). Data presented as means ± S.D. *P < 0.05 and **P < 0.01 compared to 0% blood concentration using Bonferroni-adjusted Wilcoxon signed-rank test. DST, dexamethasone suppression test.

concentrations, confirming previous studies of liquorice-induced 'pseudohyperaldosteronism' through inhibition of 11β-HSD2 with increased concentrations of cortisol in the kidneys acting on mineralocorticoid receptors (33, 36, 37, 38, 39).

Hydrocortisone cream on the fingers used to handle the Salivette® swab resulted in extremely high salivary cortisol concentrations (1000-fold increase) but only slightly elevated salivary cortisone concentrations (1.3-fold increase) and thus profoundly elevated cortisol:cortisone ratios, in line with previous findings (40). The very large effect of topical hydrocortisone cream in this experimental situation would not be likely to deceive any endocrinologist into a CS diagnosis, especially with the ability to discard the sample based on the high cortisol:cortisone ratio. However, the magnitude of effect observed in this study indicates that even slight contamination with topical hydrocortisone, e.g. hours after self-application of or touching another person's skin with hydrocortisone cream can result in significantly elevated salivary cortisol. Our results support disregarding saliva cortisol samples with a cortisol:cortisone ratio >1, which should be considered contaminated with topical hydrocortisone (21). Measures to avoid hydrocortisone cream contamination of the

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saliva swab/sampling include using nitrile gloves during the self-sampling procedure or saliva sampling without handling the swab with the fingers. In relation to the profound effect on salivary cortisol by contamination with hydrocortisone cream, salivary cortisone was only slightly increased. Possible causes include traces of cortisone in the hydrocortisone cream used, 11β-HSD2 enzymatic activity in the saliva converting some hydrocortisone into cortisone, and 11β-hydroxysteroid dehydrogenase type 1 enzyme on the skin converting excess cortisol to cortisone in the samples through its bidirectional nature (41, 42).

We found that contamination of the saliva sample with ≥0.5% blood significantly increased salivary cortisol concentrations and should therefore be rejected from the analysis of salivary cortisol. Visual examination of the saliva sample showed a high sensitivity and specificity for identifying samples with ≥0.5% blood contamination. Conversely, salivary cortisol:cortisone ratio ≥1 was found in only a few of the samples contaminated with ≥0.5% blood and can thus not be used as a marker to identify samples for rejection due to blood contamination.

An important finding is that concentrations of salivary cortisone were not affected by either liquorice intake or blood contamination and were only marginally affected by topical hydrocortisone contamination. Together with our earlier finding of a significantly higher diagnostic accuracy for late-night salivary cortisone vs LNSC for detecting CS (5), we propose salivary cortisone as a more robust biomarker for screening CS than LNSC.

CV_A for salivary cortisol and cortisone were comparable to previously presented results for LC-MS/MS methods (43). However, our calculated CV_I and CV_G for LNSC are higher than reported in a previous study with salivary cortisol analyzed using a modified RIA (44). Also, morning salivary cortisol CV_I and CV_G were higher in our study than previously reported in a study using Roche Elecsys (45). Notably, we did not exclude outliers as in these earlier studies. Interestingly, CV_I and CV_G for salivary cortisone were lower than for salivary cortisol.

A limitation of this study is the low number of participants, especially for the separate liquorice dose groups. However, we did observe clinically relevant increases in salivary cortisol concentrations with all three preanalytical confounders and indicate the means to avoid these. Another limitation is that only healthy individuals within a limited age span were included. Thus, whether the observed effects of liquorice intake upon 11β-HSD2 enzymatic activity and subsequent salivary cortisol concentrations are the same in patients with CS and in older age groups requires further studies. Furthermore, in this study, participants were instructed to consume the liquorice between 18:00 and 21:00 h with at least 90 min of fasting before intake. Whether the observed effect on salivary cortisol is similar to other consumption patterns in relation to time of day, food intake, and saliva sampling requires further studies.

In conclusion, salivary cortisol may be falsely elevated by liquorice, blood in saliva, and topical hydrocortisone. These confounders may be minimized by refraining from liquorice consumption for 1 week before sample collection, avoiding handling sampling equipment with bare hands, and rejecting samples with a visible pink tint or a cortisol:cortisone ratio ≥1. Salivary cortisone measured by LC/MS-MS has a high diagnostic accuracy for CS and is essentially unaffected by the aforementioned preanalytical confounders and may thus be considered a more robust screening instrument for CS than salivary cortisol.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EC-22-0324.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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