

# Scalp hair cortisol for diagnosis of Cushing's syndrome

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## ABSTRACT

**Objective:** Current first-line screening tests for Cushing's syndrome (CS) only measure time-point or short-term cortisol. Hair cortisol content (HCC) offers a non-invasive way to measure long-term cortisol exposure over several months of time. We aimed to evaluate HCC as a screening tool for CS.

**Design:** case-control study in two academic referral centers for CS

**Methods:** Between 2009 and 2016, we collected scalp hair from patients suspected of CS and healthy controls. HCC was measured using ELISA. HCC was available in 43 confirmed CS patients, 35 patients in whom the diagnosis CS was rejected during diagnostic work-up and follow-up (patient controls), and 174 healthy controls. Additionally, we created HCC timelines in two patients with ectopic CS.

**Results:** CS patients had higher HCC than patient controls and healthy controls (geometric mean 106.9 vs 12.7 and 8.4 pg/mg, respectively,  $P < 0.001$ ). At a cut-off of 31.1 pg/mg, HCC could differentiate between CS patients and healthy controls with a sensitivity of 93% and a specificity of 90%. With patient controls as a reference, specificity remained similar (91%). Within CS patients, HCC correlated significantly with urinary free cortisol ( $r = 0.691$ ,  $P < 0.001$ ). In two ectopic CS patients, HCC timelines indicated that cortisol was increased 3 and 6 months before CS became clinically apparent.

**Conclusions:** Analysis of cortisol in a single scalp hair sample offers diagnostic accuracy for CS similar to currently used first line tests, and can be used to investigate cortisol exposure in CS patients months to years back in time, enabling estimation of disease onset.

## INTRODUCTION

Glucocorticoids are a class of steroid hormones that are produced under the influence of the hypothalamus-pituitary-adrenal (HPA) axis and have mediating effects in metabolism, inflammation, circulation and behavior. Cushing's syndrome (CS) occurs when there is an excess of glucocorticoids, either from an exogenous source or an excessive endogenous production of cortisol. Endogenous CS is a rare disorder, most commonly caused by an ACTH producing pituitary adenoma (Cushing's disease), less frequent are primary adrenal causes and ectopic CS [1]. Ectopic CS results from secretion of adrenocorticotrophic hormone (ACTH) from a non-pituitary source, or very rarely, from ectopic corticotropin-releasing hormone (CRH) secretion. Ectopic CS is often associated with higher ACTH levels than in Cushing's disease, leading to more fulminant CS [2]. While a number of signs and symptoms are deemed highly suggestive for CS (e.g. facial plethora, proximal muscle weakness, purple striae and easy bruising), many are non-specific and highly prevalent in the general population, such as metabolic syndrome features, osteoporosis and depression [3]. This overlap with common chronic conditions is likely to cause delay in the diagnosis of CS in many cases.

Diagnosis of CS is further complicated by the fact that no single biochemical test for cortisol exposure offers perfect diagnostic accuracy. First-line tests for CS that are recommended by the Endocrine Society's guideline are urinary free cortisol (UFC) in 24 hour urine collections, late-night salivary cortisol (LNSC) and the 1 mg overnight dexamethasone suppression test (DST) [3]. Cortisol secretion can be variable in CS, illustrated by the fact that there is high variability of UFC in patients with active CS [4] and that many patients have at least one normal UFC [5]. In general, multiple tests are needed to establish a diagnosis, and thus, UFC and LNSC measurements both are usually performed on at least two different occasions [3].

Over the past few years there is an increasing use of cortisol in scalp hair as a measure of long-term cortisol exposure [6]. Hair cortisol content (HCC) has been used to investigate long-term cortisol levels in association with cardiovascular disease [7-9], obesity [10-12] and metabolic syndrome [13]. We and others have previously reported increased HCC in a small number of CS patients, including cases of cyclic CS [14, 15]. HCC has practical advantages over currently used diagnostic tests, since sample collection can easily be performed in an outpatient setting and is not dependent on patient adherence to sampling instructions. Furthermore, HCC measurement offers retrospective information about cortisol levels over months of time in a single measurement, thereby potentially circumventing the limitations posed by the variability in cortisol secretion in endogenous CS [6].

We aimed to establish the optimal cut-off value of HCC for the diagnosis of endogenous CS. In order to do this, we measured HCC in patients with confirmed CS, in healthy controls, and in patients who were initially suspected to have CS but in whom CS was excluded during work-up. Furthermore, we aimed to explore the potential of HCC to retrospectively assess the onset of hypercortisolism in severe (ectopic) CS.

## SUBJECTS AND METHODS

### Patients and controls: HCC for diagnosis of Cushing's syndrome

Between 2009 and 2016, patients who visited the endocrinology outpatient clinic at a single academic medical center (Erasmus MC, Rotterdam, The Netherlands) and were suspected of CS were requested to take part in the present study. Within this period, we included all patients who were evaluated for Cushing's syndrome and consented to take part in the present study, and in whom Cushing's syndrome could either be confirmed, or excluded based on diagnostic tests and the clinical evaluation of the treating endocrinologist. Part of these patients were included in a previously published case series [15]. We excluded patients who used topical hydrocortisone, as well as users of systemic corticosteroids. Patients were classified as having endogenous CS if hypercortisolism could be biochemically confirmed in the three months before study inclusion (using UFC, DST and/or LNSC), and the cause of hypercortisolism could be demonstrated. For Cushing's disease, the combination of hypercortisolism with an inferior petrosus sinus sampling showing a central to peripheral ACTH gradient, or a pituitary adenoma > 6 mm on MRI was considered diagnostic. For adrenal Cushing's syndrome, histopathology of a cortisol-producing adenoma was considered diagnostic. For ectopic Cushing's syndrome, histopathology of an ACTH or CRH producing tumor was considered diagnostic. Healthy individuals from our previously published validation study, which were included from August 2009 through April 2010, served as controls [16]. From all participants, written informed consent was obtained. This study was approved by the institutional review board of Erasmus MC. In all participants, we collected questionnaires about hair characteristics which have been shown to influence hair cortisol, which include hair washings, the use of hair products, and hair treatments like coloring and bleaching. Furthermore, this questionnaire included a question about the recent use of corticosteroids, including topical corticosteroids [6].

### Patients: HCC timelines in ectopic Cushing's syndrome

Two patients with ectopic CS and long hair were recruited at the Klinikum der Ludwig-Maximilians-Universität München (Munich, Germany) with the objective to create retrospective timelines of cortisol exposure. From both participants, written informed

consent was obtained. This study was approved by the institutional review board of the Klinikum der Ludwig-Maximilians-Universität München.

### **Measurement of hair cortisol concentrations**

In all participants we cut a scalp hair sample of approximately 150 hairs at the posterior vertex, as close to the scalp as possible. Hair processing and analysis was performed as described previously [16]. Depending on the length of the hair, from the most proximal 1-3 cm at least 10 mg of hair was weighed. For the creation of timelines in patients with ectopic CS, the entire length of hair samples was divided into segments of 1 cm length, corresponding to cortisol exposure during periods of 1 month [17]. Depending on the quantity of hair, more distal hair was divided in 2 cm segments, corresponding to 2 month periods.

After weighing, the hair was finely cut using scissors. We extracted cortisol from the hair in 1 ml of methanol during 16 hours at 52 degrees Celsius. After extraction, the methanol was transferred into clean glass tubes, evaporated under nitrogen stream, and the residue was reconstituted in 250 microliter of phosphate buffered saline (pH 8.0). We then vortexed the samples and analyzed them using a commercially available ELISA kit for cortisol in saliva (SLV-2930, DRG Instruments GmbH, Marburg, Germany). We previously determined the intra- and inter-assay variations. For the intra-assay variation, coefficients of variance (CV) were 3.1% at 4.4 ng/ml, 2.3% at 21.3 ng/ml, and 2.6% at 35.0 ng/ml. The inter-assay CVs were 7.0, 2.3, and 8.2%, respectively [18].

### **Measurement of cortisol concentrations in urine, serum and saliva**

Twenty-four hour UFC was measured on two consecutive days as a part of the routine diagnostic procedure for CS. UFC was measured using either one of two in-house methods: chemiluminescence immunoassay using unextracted urine (Immulite XPi, Siemens AG, Munich, Germany) or liquid chromatography / tandem mass spectrometry (LC/MS-MS, Waters Xevo-TQ-S, Milford, MA). The upper limits of normal of these assays are 850 (validated for cortisol production rate) and 133 nmol/24h, respectively.

Serum cortisol was measured using chemiluminescence immunoassay (Immulite XPi, Siemens AG, Munich, Germany). A morning cortisol after 1 mg dexamethasone overnight of more than 50 nmol/L suggested hypercortisolism. Salivary cortisol was measured using ELISA (SLV-2930, DRG Instruments GmbH, Marburg, Germany, or DES6611, Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany). For LNSC, a cut-off value of 9.3 nmol/L was used, as reported previously [19].

In the two patients with ectopic CS from the Klinikum der Ludwig-Maximilians-Universität München, serum cortisol was measured using Solid Phase Antigen linked Technique (Liaison, DiaSorin Deutschland GmbH, Dietzenbach, Germany), and salivary cortisol was measured using a luminescence immunoassay (Cortisol Luminescence Immunoassay, IBL International GmbH, Hamburg, Germany). UFC was measured using two different chemiluminescence immunoassays for patient A (DVIA Centaur, Siemens AG, Munich, Germany) and patient B (Liaison, DiaSorin Deutschland GmbH, Dietzenbach, Germany).

### Statistical analysis

IBM SPSS Statistics version 21 (IBM Corp., Armonk, NY) and GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA) were used for statistical analysis. Baseline characteristics were analyzed using Chi square, Mann-Whitney U and Kruskal-Wallis tests. Cortisol values were logarithmically transformed to obtain a normal distribution. HCC is expressed as a geometric mean and 95% confidence interval, in pg/mg hair. We compared HCC between CS patients, healthy controls and non-CS patients with analysis of (co)variance. To determine cut-off values and sensitivity and specificity, we created receiver operating characteristic (ROC) curves. Correlations between HCC and first-line screening tests in CS patients were analyzed using Pearson's correlation.

## RESULTS

### Diagnosis accuracy of HCC in CS

We included 43 patients with confirmed endogenous CS, including patients from a previously published case series [15]. In addition, we identified 35 patients who had suspected CS, but in whom CS diagnosis was excluded during diagnostic work-up (patient controls: median follow up 3.5 months, range 0.5 – 91.2). All 35 patient controls had UFCs below the upper limit of normal. LNSC values were measured in 33 out of 35 patient controls, and were all below the cut-off value of 9.3 nmol/L. Patients with CS were on average older than the 174 healthy controls (median age 50 [range 15 - 76] vs. 32 years [range 18 – 63],  $P < 0.001$ ) and had a higher BMI (29.4 [range 18.3 – 81.6] vs. 23.5 kg/m<sup>2</sup> [range 16.9 – 43.3],  $P < 0.001$ , Table 1). CS patients had lower BMI than patient controls (29.4 [range 18.3 – 81.6] vs. 35.2 [range 21.4 – 46.6] kg/m<sup>2</sup>,  $P = 0.011$ ) and were significantly older (50 [range 15 - 76] vs 38 [15 – 79 years],  $P = 0.009$ ). The group of healthy controls consisted of most men, followed by CS patients and patient controls (43 vs. 30 vs. 17%,  $P = 0.011$ ). The three groups were similar in terms of hair characteristics (Table 1). HCC levels were highest in CS patients (geometric mean 106.9 pg/mg, 95% CI 77.1 – 147.9,  $F(2,249) = 88.9$ ,  $P < 0.001$ ), and significantly higher than in healthy controls (8.4 pg/mg, 95%CI 7.0 – 10.0,  $P < 0.001$ ), and patients controls (12.7 pg/mg, 95% CI 8.6 –

**Table 1.** Baseline characteristics and hair cortisol concentrations

	Healthy controls N=174	Patient controls N=35	CS patients N=43	<i>P</i> <sub>diff</sub>
<i>Patient characteristics</i>				
Male (n, %)	74 (43%)	6 (17%)	13 (30%)	0.011
Age [years] (median, range)	32 (18 - 63)	38 (15 - 79)	50 (15 - 76)	<0.001
BMI [kg/m <sup>2</sup> ] (median, range)	23.5 (16.9 - 43.3)	35.2 (21.4 - 46.6)	29.4 (18.3 - 81.6)	<0.001
<i>Hair characteristics</i>				
Hair washing ≥3 per week (yes/no, %)	127/45 (74%)	25/9 (74%)	24/16 (60%)	0.209
Use of hair products (yes/no, %)	84/89 (49%)	17/17 (50%)	12/28 (30%)	0.091
Hair coloring* (yes/no, %)	36/138 (21%)	13/21 (38%)	10/33 (23%)	0.088
Hair bleaching* (yes/no, %)	13/161 (7%)	4/30 (12%)	1/42 (2%)	0.270
<i>Hair cortisol concentrations</i>				
Non-adjusted [pg/mg hair] (geometric mean, 95%CI)	8.4 (7.0 - 10.0)	12.7 (8.6 - 18.6)	106.9 (77.1 - 147.9)	<0.001
Adjusted model 1† [pg/mg hair] (geometric mean, 95%CI)	8.3 (6.7 - 10.3)	13.8 (8.6 - 21.8)	95.8 (66.6 - 137.6)	<0.001
Adjusted model 2‡ [pg/mg hair] (geometric mean, 95%CI)	8.6 (6.9 - 10.6)	15.2 (9.5 - 24.1)	93.0 (63.7 - 135.6)	<0.001
<i>First-line screening tests</i>				
Urinary free cortisol (ULN) (geometric mean, 95%CI)		0.42 (0.31 - 0.57)	3.81 (2.87 - 5.05)	<0.001
Late-night salivary cortisol (nmol/L) (geometric mean, 95%CI)		2.2 (1.4 - 3.5)	27.7 (18.1 - 42.4)	<0.001
DST cortisol (nmol/L) (geometric mean, 95%CI)		40 (30 - 54)	476 (369 - 615)	<0.001

Differences in baseline characteristics were analyzed using Chi square and Kruskal-Wallis tests. Differences in hair cortisol concentrations were analyzed using analysis of (co)variance.

BMI, body mass index; CI, confidence interval; CS, Cushing's syndrome; DST, dexamethasone suppression test; ULN, upper limit of normal.

\*Hair coloring and bleaching in the 3 months prior to hair collection.

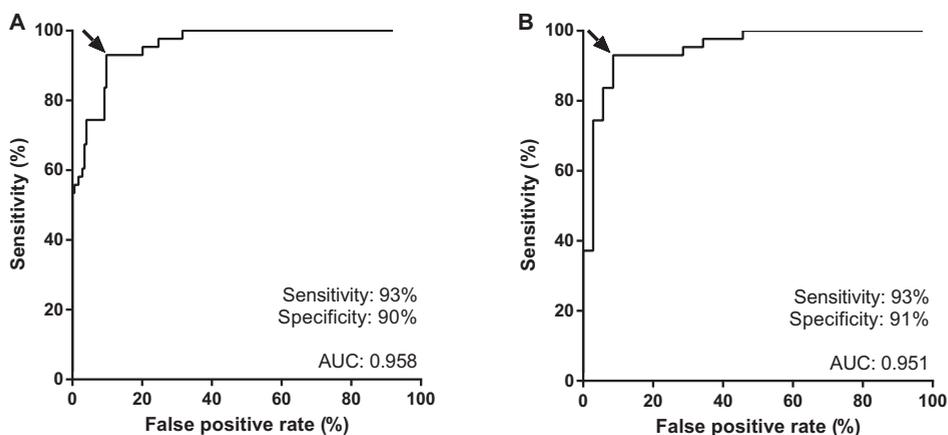
†Model 1 was adjusted for age, sex and BMI.

‡Model 2 was adjusted for age, sex, BMI and hair characteristics.

Hair cortisol data for healthy controls, and part of the CS patients have been published previously [15, 16].

18.6,  $P < 0.001$ ). Adjustment for age, sex and BMI and hair characteristics did not change these results (Table 1). In healthy controls, HCC was not significantly influenced by sex ( $t(172)=1.05$ ,  $P=0.293$ ), age (Pearson's  $r$  0.65,  $P=0.395$ ), or BMI (Pearson's  $r$  0.111,  $P=0.180$ ).

The ROC curve revealed an optimal cut-off for the diagnosis of CS of 31.1 pg/mg hair, when healthy controls were used as a reference population. For this cut-off, sensitivity and specificity were 93 and 90%, respectively (AUC=0.958, Figure 1A). When we used patient controls as a reference, the optimal cut-off for the diagnosis was the same as with healthy controls. In this analysis, sensitivity and specificity remained similar at 93% and 91%, respectively (AUC=0.951, Figure 1B).

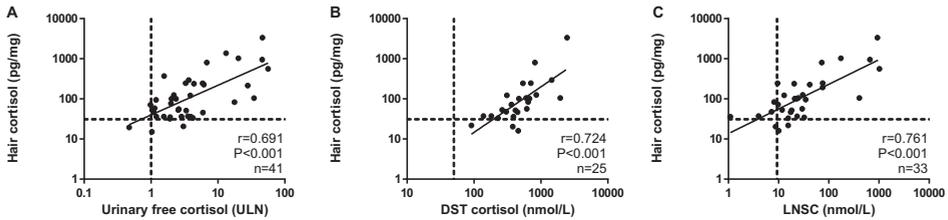


**Figure 1.** ROC curves of HCC for the diagnosis of CS, with healthy controls (A) or patient controls (B) as a reference population.

The arrows indicate a cut-off value of 31.1 pg/mg. AUC, area under the curve. \*\* $P < 0.01$ . Hair cortisol data for healthy controls, and part of the CS patients have been published previously [15, 16].

In CS patients, HCC levels significantly correlated with UFC (available in  $n=41$ , Pearson's  $r=0.691$ ,  $P < 0.001$ , Figure 2). HCC also correlated with serum cortisol after 1 mg DST ( $n=25$ ,  $r=0.724$ ,  $P < 0.001$ ) and with LNSC ( $n=33$ ,  $r=0.761$ ,  $P < 0.001$ ).

Most CS patients had Cushing's disease ( $n=26$ , 60%), followed by adrenal CS ( $n=10$ , 23%) and ectopic ACTH secretion ( $n=7$ , 16%). HCC was significantly higher in patients with ectopic ACTH secretion (geometric mean 412.5 [95%CI 176.7 – 961.1] pg/mg) compared to patients with Cushing's disease and adrenal CS (82.6 [95%CI 53.0 - 128.6] and 80.5 [95%CI 39.2 – 164.3] pg/mg, respectively.  $F(2,40)=6.18$ ,  $P=0.005$ , both pairwise comparisons versus ectopic ACTH secretion  $P < 0.01$ , Figure 3). Within Cushing's disease patients, the 6 patients with a macroadenoma had a significantly higher HCC than the 20 patients with a microadenoma (227.2 [98.2 – 524.2] vs 60.9 [44.8 – 82.6] pg/mg,  $t(24)=3.63$ ,  $P=0.001$ ).

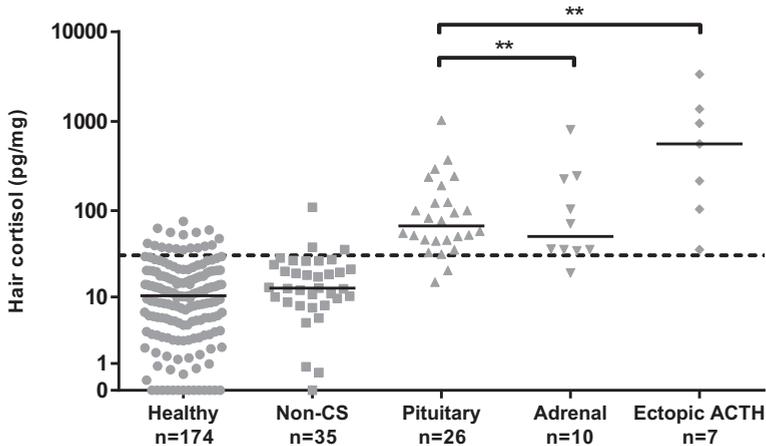


**Figure 2.** Correlation between hair cortisol and first-line diagnostic tests in Cushing's syndrome patients. Panel A, urinary free cortisol (UFC). Panel B, dexamethasone suppression test (DST). Panel C, late-night salivary cortisol (LNSC). Hair cortisol content is expressed on a logarithmic scale, in pg/mg hair. UFC is expressed in times upper limit of normal (ULN) on a logarithmic scale. The horizontal dotted line represents the cut-off value for hair cortisol of 31.1 pg/mg. The vertical dotted line represents the upper limit of normal for first-line diagnostic test. R, Pearson's correlation. Hair cortisol data for healthy controls, and part of the CS patients have been published previously [15, 16].

### Hair cortisol timelines in patients with ectopic CS

Patient A was a 54-year old woman who presented at the Klinikum der Ludwig-Maximilians-Universität in Munich in October 2014 with severe hypokalemic hypertension (minimum serum potassium 1.8 mmol/L) and edema, for evaluation of mineralocorticoid excess. However, plasma renin concentration (2 mU/L, normal 4.4 – 46) and plasma aldosterone (< 83 pmol/L, normal 139-979 pmol/L) were suppressed. Before October 2014, she was asymptomatic. Classical signs and symptoms of CS were absent. ACTH-dependent hypercortisolism was found, with a UFC of 44358 nmol/24h (normal < 414 nmol/24h), and a baseline serum cortisol of 3862 nmol/L (normal 138 – 690 nmol/L). The patient underwent CRH stimulation testing, low and high dose dexamethasone testing, and sinus petrosus inferior sampling suggesting an ectopic ACTH source, but intensive imaging studies including FDG PET, DOTATATE PET and DOPA PET scanning were negative. The patient underwent emergency bilateral adrenalectomy after controlling hypercortisolism by CYP11B2 blockade using intravenous etomidate. The source of ACTH has not been detected thus far (last follow-up June 2016).

In the middle of November 2014, approximately 4 weeks after the onset, a hair sample was obtained, with a length of 26 cm, allowing us to retrospectively assess cortisol levels for over 2 years back in time (26 months; Figure 4A). The most proximal 12 cm of hair, which correspond with the period from November 2013 to November 2014, were divided into 1 cm segments. The most distal 14 cm of hair were divided into 2 cm long segments, spanning the period between September 2012 and November 2013. Between April 2013 and April 2014, HCC values in Patient A were around the previously established cut-off value (<31.1 pg/mg). From April 2014 onwards, 6 months before clinical presentation, a gradual rise in HCC can be observed, with a maximum of 78.2 pg/mg. In the hair seg-



**Figure 3.** Hair cortisol concentrations in healthy controls, patients without Cushing's syndrome (Non-CS patients), and in patients with Cushing's syndrome

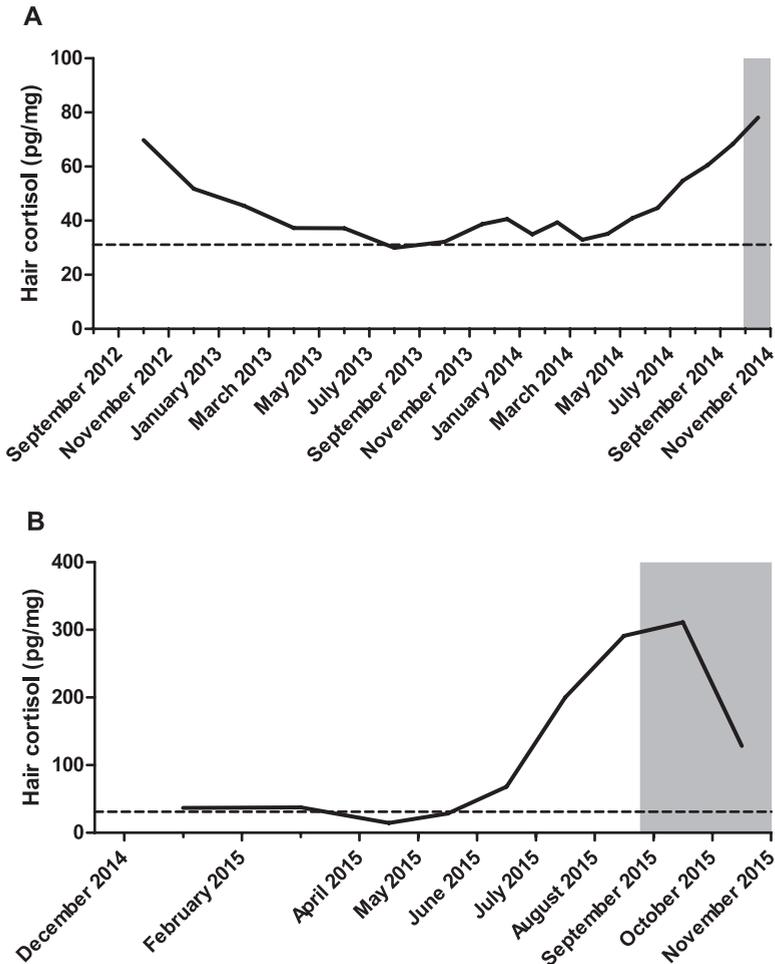
Hair cortisol values are presented on a semi-logarithmic scale. Values in patients with Cushing's syndrome are stratified by etiology. Solid horizontal lines represent group medians, the dotted horizontal line represents the cut-off value of 31.1 pg/mg. Hair cortisol data for healthy controls, and part of the CS patients have been published previously [15, 16].

ments corresponding to the fall of 2012, increased HCC can be observed as well (Figure 4A). Patient A did not recall any symptoms of CS during this period.

Patient B was a 59-year old woman who presented in September 2015 at the Klinikum der Ludwig-Maximilians-Universität in Munich with symptoms of rapid weight gain (7 kg in 2 weeks), edema, abdominal obesity, plethora, hirsutism, hair loss, proximal muscle weakness, irritability and polydipsia. All symptoms had a recent onset of less than one month earlier. Clinical examination showed plethora, dorsal fat pad, proximal muscle weakness, edema of the lower extremities, moon face, abdominal obesity, hirsutism, alopecia and dry skin. ACTH dependent hypercortisolism was found, with a LNSC of 308 nmol/L (normal <4.1 nmol/L), and a UFC of 11361 nmol/24h (normal < 229 nmol/24h). A CRH test, high-dose dexamethasone suppression test and inferior sinus petrosus sampling were all in accordance with an ectopic Cushing's syndrome. On PET-CT, a suspected pancreatic lesion was seen, but this was not evident from an abdominal MRI and endoscopic sonography that followed. Based on a diagnosis of occult ectopic Cushing's syndrome, adrenostatic therapy was started in December 2015, with ketoconazole and metyrapone and hydrocortisone substitution. After this, the patient became normocortisolemic. In June 2016, UFC was 151 nmol/24h (normal < 229/24h).

In November 2015, slightly over 2 months after the onset of symptoms, a hair sample of 11 cm length was obtained, allowing us to create a retrospective timeline spanning De-

ember 2014 to November 2015 (Figure 4B). The most proximal 7 cm of hair was divided into 1 cm long segments, while the most distal 4 cm was divided into two 2 cm long segments. In hair segments corresponding to December 2014 to June 2015, cortisol levels appear below or just above the cut-off level (<31.1 pg/mg). From June 2015, 3 months before the onset of symptoms, cortisol levels rose sharply, up to 311.2 pg/mg.



**Figure 4.** Hair cortisol timelines in patients with a recent onset of ectopic Cushing's syndrome. The horizontal dotted line represents the cut-off value of 31.1 pg/mg. The grey area corresponds to period in which the patient had signs and/or symptoms of Cushing's syndrome.

## DISCUSSION

In the present study, we used HCC to distinguish between individuals with CS, and those without. We found that a cut-off value of 31.1 pg/mg discriminates well between CS and healthy controls. Additionally, we showed that this test performs equally well when CS patients are compared to patients who were suspected to have CS, but in whom the diagnosis could be excluded (patient controls). The sensitivity of 93% and specificity 90% (healthy controls) and 91% (patient controls) we found are well in line with current first-line tests for CS as reported in recent meta-analyses: UFC (mean sensitivity and specificity of 84 and 92%, respectively) [20], LNSC (95 and 92%) [21] and 1 mg DST (99 and 88%) [20]. However, it must be noted that the performance of first-line test differs significantly between studies.

In two patients with recent onset of ectopic CS, we created retrospective timelines of cortisol exposure. In both cases an increase can be seen months before the effects of hypercortisolism became clinically evident. In the case of patient A, the hair sample surprisingly seems to indicate hypercortisolism as much as 2 years before hair collection. This may indicate that she has suffered from a previous period of hypercortisolism and thus a cyclical form of CS, although the patient did not recall symptoms of CS around that period. We previously showed that hair cortisol can well reveal cyclical forms of Cushing's with more than one period of symptomatic hypercortisolism in the past [15]. Care must be taken not to draw exaggerated conclusions based on hair cortisol timelines, especially in lack of supporting evidence. However, these cases do illustrate the potential of HCC measurements, offering the unique possibility to retrospectively assess cortisol levels months up to years back in time [6]. The use of HCC as a historical record of Cushing's syndrome was first shown by Thompson *et al.* [14], and we have published additional examples in which HCC timelines corresponded well with the clinical course in Cushing's [15]. Now, for the first time we showed that the onset of hypercortisolism due to ectopic ACTH production could be estimated. From this, we may learn in the future how the duration of the disease (before diagnosis) may affect the somatic and neuropsychological outcomes of the disease both in the short term, and, importantly, also in the long term after cure.

Besides the addition of retrospective long-term cortisol measurement, scalp hair cortisol offers significant advantages over current first-line tests for hypercortisolism. First, hair sampling can easily be performed in an inpatient or outpatient setting. Unlike all of the other first-line tests, it does not depend on the adherence of patients to instructions concerning sample collection or medication use. Obviously, complete baldness makes scalp hair sample collection impossible, but in our experience, even in individuals with

little scalp hair a useful sample can be obtained successfully. Second, our results are based on collecting just one hair sample. Currently, often multiple urine or saliva collections are required to establish the diagnosis of CS [3, 5]. Recently, it has been shown that HCC in the proximal 1 cm of scalp hair (representative of the month before sample collection) correlates well with integrated daily salivary cortisol, based on three daily saliva samples which were collected for a 30-day period [22]. This is strong evidence that cortisol in hair truly reflects the average long-term exposure to cortisol, which dampens the effects of the incidental fluctuations (such as stress at the moment of sample collection) that seriously affect the concentrations that are measured in the other tests.

Although measurement of scalp hair cortisol are more laborious than cortisol measurements in other matrices, due to hair sample preparation and extraction, it requires no sophisticated techniques. Hair cortisol measurements have been performed for years by several expertise centers [14, 16, 23]. A previously published round robin comparison between hair cortisol methods from different labs found that different methods correlated well, with  $r$  squared values between 0.88 and 0.97 [24]. Further standardization of analytical procedures, as well as automation of hair sample work-up may help routine laboratories set up hair cortisol assays. Like any analytical method hair cortisol relies on proper training of laboratory personnel, in order to limit operator variability. Furthermore, when using any immunoassay based method for an extended period of time, there is a risk of assay performance drift. In our lab, we have consistently used the same equipment and ELISA kits. However, we do rely on quality controls procedures as performed by the manufacturing company.

Several limitations have to be considered when interpreting our data. We included patients in our study, who either had unequivocally confirmed CS, and patients in whom it could unequivocally be excluded. Since the diagnosis of CS was based on current first-line tests, our data do not allow a comparison between the diagnostic accuracy of HCC and these other tests. Additionally, we did not investigate the added value of hair cortisol in cases in which the diagnosis of CS remains unclear. Future prospective studies may show the additional value of HCC in these cases.

Experience with current first-line tests for CS illustrates the importance of investigating possible confounders of screening tests for CS. UFC may be falsely elevated due to high urine volumes and stress, and falsely low with impaired renal function. The DST is subject to the absorption and metabolism of dexamethasone, which can be influenced by medication use. Furthermore, because total levels of cortisol are measured in serum, the DST is often false positive when medication is used that increases the levels of corticosteroid binding globulin, such as oral contraceptives. LNSC can be falsely elevated

due to stress, an altered day rhythm (e.g. in shift workers) and contamination by blood or corticosteroid containing products [3]. In our study we did not include any patients who used topical hydrocortisone. However, this is likely to be a confounder which should be taken into account when interpreting hair cortisol concentrations, similar to LNSC [25]. Since HCC estimates cortisol over a much larger timeframe than other tests, the influence of acute stress can be assumed to be diminished.

Just like with measurements in other matrices, many conditions that slightly increase cortisol in scalp hair have now been described. These include depression and obesity [10-12, 23], which are also common features seen in CS. Therefore, we believe it remains of vital importance that the use of HCC for the diagnosis of CS is limited to populations with a high *a priori* probability. As we have shown in our comparison with patient controls, HCC performed well at diagnosing CS in a population where the suspicion for this diagnosis was high. However, not all factors contributing to HCC may be known yet. Furthermore, in the current study we did not have data on ethnicity, which has been reported to slightly influence hair cortisol [26].

In conclusion, our results indicate that measuring cortisol in a single sample of scalp hair offers a high sensitivity and specificity for the diagnosis of Cushing's syndrome (CS). Together with a straightforward sample collection procedure, this method may prove to be a convenient non-invasive screening test for CS. Additionally, our results indicate that hair cortisol measurements provide clinicians a tool to retrospectively assess cortisol secretion in patients with CS, months to years back in time. This also offers the opportunity to estimate the onset of hypercortisolism and thus the duration of the disease before diagnosis. Future studies using retrospective timelines of hair cortisol may lead to more insight into the question how total duration of hypercortisolism may affect both somatic and neuropsychological outcomes of the disease in the short and long term.

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