

# Hair cortisol concentration in a population without hypothalamic–pituitary–adrenal axis disorders

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## Abstract

**Background.** Measuring hair cortisol seems to be a good alternative to laboratory tests used thus far in routine endocrine diagnostics, primarily because it is independent of the circadian rhythm of cortisol. Due to the average hair growth of 1 cm per month, the results are related to the average blood cortisol levels over the previous weeks, months or years (depending on the length of the hair sample).

**Objectives.** The aim of this study is an attempt to apply hair cortisol concentration (HCC) measurements to clinical endocrine diagnostics, based on reference cortisol concentrations in the blood in a population without disorders of the hypothalamic–pituitary–adrenal axis (HPA).

**Material and methods.** In the final selection process, 44 patients were enrolled in the study, all with negative interviews regarding disorders of the HPA and with reference levels of cortisol concentration obtained in routine laboratory tests. In the pre-analytic phase, we used 1 cm proximal hair strands cut from the posterior vertex area of the head, followed by the incubation of a 20 mg hair sample in methanol. The final cortisol measurement was done using an enzyme-linked immunosorbent assay (ELISA).

**Results.** The results of HCC ranged from 2 pg/mg up to 51.63 pg/mg. The diurnal decrease in cortisol levels was significantly lower in females than in males ( $p = 0.031$ ), but we do not consider that difference to be clinically significant. The difference in the HCC between males and females was not statistically significant ( $p = 0.767$ ). The linear regression coefficient for age was not statistically significant ( $p = 0.847$ ). Neither the regression coefficients for gender nor the gender and age interactions were statistically significant ( $p = 0.815$ ).

**Conclusions.** Hair cortisol concentration measurement, unlike other endocrinological tests, gives information about the cortisol concentration in the long-term perspective. The results obtained in this study may be used as a reference for further research aimed at determining normal values of HCC.

**Key words:** measurements, validity, hair cortisol, reference

## Cite as

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## Introduction

The aim of this study was an attempt to apply measurements of cortisol concentrations in hair to clinical endocrine diagnosis, based on reference cortisol concentrations in the blood in a population with no disorders of the hypothalamic–pituitary–adrenal axis (HPA).

Routine endocrine diagnostics for patients with HPA axis disorders entail a series of blood, urine and saliva tests. These include daily serum cortisol, urine free cortisol (UFC), saliva cortisol, and several hormonal dynamic tests using stimulation, such as the corticotropin-releasing hormone (CRH) stimulation test, or inhibition test, e.g., the low/high dose dexamethasone suppression test. The numerous methods of cortisol assessment arise from the necessity of collecting reliable results that correlate with a clinical picture. Their interpretation is often impeded due to variations in the method of determination, environmental factors, physiological variations in cortisol levels, drugs/xenobiotics taken, and pathological conditions secondarily affecting the HPA axis. For this reason, there is no single test that will produce a credible result, and therefore, at least a few tests must be performed to provide a basis for a proper diagnosis.<sup>1–3</sup>

Publications related to hair cortisol concentrations (HCC) mainly refer to non-primary endocrine disorders, including depressive-anxiety disorders, stressful events, shift work, physical exertion, alcohol abuse, smoking, chronic illnesses, or doping control.<sup>4–12</sup> Among the most relevant endocrine disorders in the context of HCC assays are Cushing's syndrome, Cushing's subclinical syndrome, Addison's disease, secondary adrenal insufficiency, and cortisol/hydrocortisone substitution.<sup>13–20</sup>

Measurement of HCC seems to be a good alternative to the laboratory tests used thus far in diagnosing endocrine disorders, primarily due to its independence from the circadian rhythm of cortisol. Given the average hair growth of 1 cm per month, the results are related to average blood cortisol levels over the past weeks, months or years, depending on the length of the hair sample.<sup>21–23</sup>

The presence of cortisol in hair, as well as other steroid hormones, is most probably a result of the passive diffusion of free cortisol from blood to the hair matrix during its formation in hair follicles. Another possible explanation for the presence of cortisol in hair is its presence in sweat and sebum. Hence, its concentration may depend on additional factors such as gender (stimulation of sweat and sebum production by androgens), age (slower hair growth with age), hair dyeing (possible flushing out of cortisol by chemicals, interference with HCC assays), physical activity, hygienic habits (influence of hair washing frequency), or exposure to sunlight.<sup>11,24–31</sup> Additional benefits of HCC assessment are its non-invasive sample collection procedure and the possibility of sample storage at room temperature for a long period of time without prior processing.

Hair cortisol concentration measurement is most often based on immunological methods, such as the enzyme-linked immunosorbent assay (ELISA) or liquid chromatography tandem mass spectrometry (LC-MS/MS).<sup>27,32–37</sup> Measurement of HCC in humans was initiated by J.S. Raul in 2004.<sup>36</sup> The population analyzed in his study consisted of 44 volunteers recruited on the basis of a questionnaire. The HCC measurement was done using chromatography, and the amount of cortisol in the samples ranged from 5 pg/mg to 91 pg/mg. Since the first report, a number of publications analyzing the HCC measurement have involved different populations of subjects recruited on the basis of demographic and anthropometric factors.<sup>24</sup> The reliability of HCC measurement is limited due to the heterogeneity of the populations analyzed, the various methodologies of the assays, comparative assessment without initial laboratory diagnostics regarding blood cortisol concentrations, and the lack of commonly accepted HCC reference values. An attempt to establish reference values of HCC in healthy subjects who have also been evaluated for cortisol levels using standard laboratory tests remains ongoing.

## Material and methods

We included 88 patients who were hospitalized in the Department of Endocrinology and Internal Medicine at the Medical University of Gdańsk, Poland, for the evaluation of hormone levels during the course of an incidental adrenal tumor. All participants signed a written informed consent to participate in the study. The study was approved by the Bioethical Committee of the Medical University of Gdańsk. Enrollment in the project was carried out in 2016.

The routine diagnostic protocol consisted of measurement of morning plasma adrenocorticotrophic hormone (ACTH) (ACTH8am), morning plasma cortisol (MPC8am), evening plasma cortisol (EPC8pm), 24-hour urinary free cortisol excretion (UFC24h), and the 1 mg overnight dexamethasone suppression test (1mgONDST). These measurements were performed at the Central Laboratory of the University Hospital in Gdańsk. Patients taking glucocorticosteroids during the preceding year and those with conditions likely to affect the results of the tests – in particular depression, alcoholism, acute/chronic inflammatory disease, and primary or secondary HPA disorders – were excluded from the study. In the final selection process, 44 patients were enrolled in the study, all with reference levels of ACTH8am (reference: <46 pg/mL), MPC8am (reference: 101–535 nmol/L), EPC8pm (reference: 79–478 nmol/L), UFC24h (reference: 12–486 nmol/24 h), a regular diurnal decrease of cortisol (DDC; reference: EPC < MPC more than 50%), and proper suppression of cortisol after 1mgONDST (reference: <50 nmol/L).

A hair sample was collected in the examination room from the posterior vertex area of the head, using a sterile

**Table 1.** Characteristics of the population. Data is presented as mean  $\pm$  standard deviation (SD) and median  $\pm$  interquartile range (IQR)

Variable	Total	Female	Male	p-value
Number	44	31	13	n/a
Age [years]	63.3 $\pm$ 10.3	63.6 $\pm$ 8.8	62.5 $\pm$ 13.8	0.757
Morning plasma cortisol [nmol/L]	338.0 $\pm$ 113.8	323.7 $\pm$ 109.4	375.0 $\pm$ 120.3	0.175
Evening plasma cortisol [nmol/L]	111.1 $\pm$ 46.8	115.2 $\pm$ 46.8	101.4 $\pm$ 47.1	0.379
Diurnal decrease of cortisol [%]	-65.5 $\pm$ 11.9	-63.0 $\pm$ 10.6	-71.4 $\pm$ 13.1	0.031
Urine free cortisol [nmol/24 h]	204.3 $\pm$ 130.2	201.7 $\pm$ 127.9	210.7 $\pm$ 140.7	0.837
ACTH [pg/mL]	29.95 $\pm$ 20.01 (22.8 $\pm$ 15.7)	27.67 $\pm$ 19.11 (22.0 $\pm$ 14.4)	35.39 $\pm$ 21.81 (26.8 $\pm$ 19.1)	0.095
1mgONDST [nmol/L]	32.27 $\pm$ 6.07 (28.0 $\pm$ 8.0)	33.23 $\pm$ 6.61 (30.0 $\pm$ 9.0)	30.00 $\pm$ 3.83 (28.0 $\pm$ 3.0)	0.1374

1mgONDST – 1 mg overnight dexamethasone suppression test; ACTH – adrenocorticotrophic hormone; n/a – not applicable.

scalpel to cut an approximate quantity of 100–200 hair strands as close as possible to the scalp. The samples obtained were stored in dry envelopes until further analysis. Next, 1 cm segments of the hair strands were cut away near the follicles, and following weighing, exactly 20 mg of the sample was used in the study. The preparation of the hair samples was performed at the Department of Clinical Nutrition and Dietetics at the Medical University of Gdańsk. First, the hair samples were placed in 10 mL plastic tubes and flushed with 2 mL of methanol. Next, they were transferred to 5 mL tubes and incubated at 50°C in 1 mL of methanol for 24 h. The extract was then transferred to 3 mL tubes, after which it was evaporated. Finally, the residue was dissolved in 250  $\mu$ L of phosphate-buffered saline (PBS) by incubation for 1 h.

The final HCC measurement was done at the Central Laboratory of the University Hospital in Gdańsk using an ELISA Cortisol Assay (IBL International GmbH, Hamburg, Germany; catalog No. RE 52611). The assay is reported to demonstrate cross-reactivity with prednisolone (30%), 11-desoxy-cortisol (7%), cortisone (4.2%), prednisone (2.5%), corticosterone (1.4%), and less than 1% with other tested substances. The limit of detection and functional sensitivity are calculated to be 0.005  $\mu$ g/dL and 0.030  $\mu$ g/dL, respectively. A detailed description of the assay is available on the manufacturer's website (<http://www.ibl-international.com/en/cortisol-saliva-elisa>). For each sample, the HCC was measured twice, and the average value was used in the statistical analysis.

Standard descriptive statistics were prepared. Conformity to a normal distribution was assessed using a histogram analysis. Some of the dependent variables underwent a logarithmic transformation before they were included in the parametric analysis. The arithmetic mean of the 2 HCC assessments was analyzed. The results of HCC are presented as pg/mg according to the cortisol concentration in the analyzed volume of the solution (250  $\mu$ L) based on the amount of incubated hair (20 mg). Comparisons in subgroups were evaluated using the t-test or the Mann–Whitney U test. A multivariate regression (general linear

analysis was used to evaluate the relationship between age and HCC. The regression model also included gender and analyzed the interaction between age and gender. P-values <0.05 were considered statistically significant. All the calculations were performed using the STATA Statistical Package v. 13.1 (StataCorp LLC, College Station, USA).

## Results

The size of the population, demographic data and the parameters of the pituitary-adrenal axis that were evaluated are presented in Table 1. The diurnal decrease in cortisol levels was significantly lower in females than in males ( $p = 0.031$ ). However, we consider that difference not to be clinically significant. There were no statistically significant differences between other variables.

The results of HCC ranged from 2 pg/mg up to 51.63 pg/mg. The difference in HCC between males and females was not statistically significant ( $p = 0.767$ ). The detailed characteristics of the HCC results are presented in Table 2. We also analyzed the relationship between age and HCC. The results are shown in Fig. 1. The linear regression coefficient for age was not statistically significant ( $p = 0.847$ ). The regression coefficients for gender and for gender and age interactions were also not statistically significant ( $p = 0.815$ ).

**Table 2.** Hair cortisol concentration (HCC) results [pg/mg]

Variable	Total (n = 44)	Female (n = 31)	Male (n = 13)
Mean	7.17	7.74	5.82
SD	10.40	12.05	4.63
Median	3.38	3.25	3.63
IQR	2.97	2.56	6.19
Minimum	2.00	2.00	2.13
Maximum	51.63	51.63	17.06

SD – standard deviation; IQR – interquartile range.

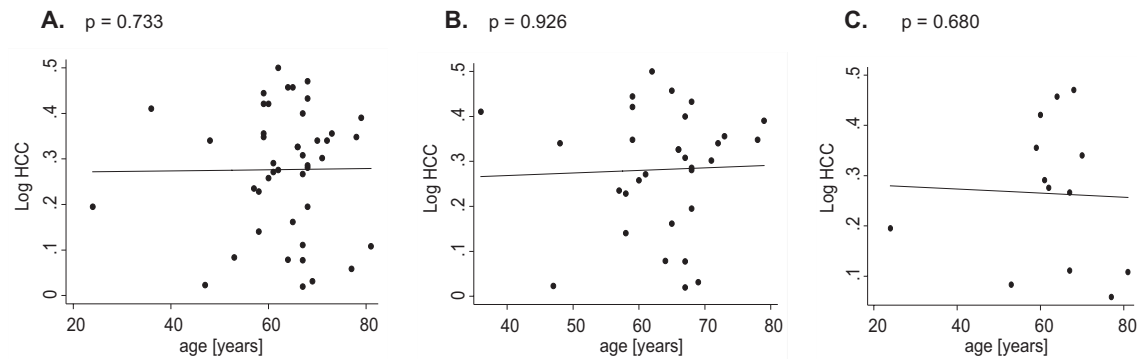


Fig. 1. Association between hair cortisol concentration (HCC) and age

A – all; B – females; C – males.

## Discussion

Hair cortisol concentration assessment is a multistep process, starting with proper hair sampling, followed by extraction of cortisol at the pre-analytical stage and concluding with final laboratory evaluation using one of the reference methods. Each of these steps can have a significant impact on the final result. Hence, the various phases of HCC assessment have been subjected to evaluation in numerous studies. In our study, the number of collected hair strands from a given individual was largely a result of a compromise between the need to obtain as much material as possible and esthetic issues, especially among the female population. In most studies, the amount of hair analyzed ranges from 2.5 mg to 150 mg, with the most widely used amount being 10–20 mg.<sup>6,7,33,34,38</sup> The optimal site for hair sampling is the vertex posterior area of the head; possible individual variation in HCC can be as high as 24%, depending on the site of collection, as demonstrated by drug and xenobiotic studies.<sup>39</sup> Incubation of a total hair sample (1-centimeter fragments) paradoxically results in higher HCC than milled hair, as it has been shown in 1 work describing HCC methodology.<sup>40</sup> Extraction of cortisol using a methanol solution is one of the most commonly used methods.<sup>4,24,41</sup> Analyses of various methods of HCC determination indicate the superiority of ELISA in terms of the sensitivity of cortisol measurement.<sup>40,42</sup> This assay is very useful due to its widespread availability as well as its ease of implementation. The correlations between HCC measurements with ELISA and LC-MS/MS in interlaboratory comparisons have been found to be strong.<sup>42</sup>

The essential problem in HCC analysis is to determine the degree of cortisol extraction from hair samples. Generally, we assumed close to 100% cortisol recovery by methanol extraction, which might not be fully true based on the latest reports. One publication has demonstrated higher extraction efficiency by methanol and acetone over methanol alone.<sup>40</sup> However, most published works used cortisol extraction by methanol as a reference method.

Unlike previous studies, our work focused on the deep characteristics of the study population. The recruited volunteers may be regarded as a reference group because of their

extensive screening for regular levels of cortisol. Thanks to the recruitment protocol, the impact of anthropometric and demographic parameters possibly affecting the HPA axis during hair collection (e.g., gender, body weight, hair color, race) has largely been excluded. We believe that higher maximal HCC in females ( $n = 31$ ,  $HCC = 51.63$  pg/mg) vs males ( $n = 13$ ,  $HCC = 17.06$  pg/mg) may possibly be due to the small male population tested and may not be related to other factors. Most studies exclude gender as a potential confounding factor in adult participants.<sup>13,27,33,36</sup> The issues outlined in Material and methods section meant that only 44 patients out of 88 met the final inclusion criteria based on the study protocol (incorrect DDC and 1mgONDST were the most common reasons for exclusion). An interview that excluded the enrollment of patients with primary or secondary HPA disorders in the period >1 year prior to sampling further strengthens our results since analyzed hair samples reflected an average cortisol concentration of past month only.

## Conclusions

The results of the study indicate the possibility of using HCC measurement in all subjects under the condition of receiving a sufficient hair sample. Cortisol extraction using a methanol medium is easy to perform with basic laboratory equipment. Commercial ELISA assays have high sensitivity and specificity with a very low cortisol detection threshold.

The study was conducted on a homogeneous group of participants who did not display corticotrophic axis disorders. The HCC ranged from 2 pg/mg to 51.63 pg/mg. The results obtained in the present work may be used as a reference for further research.

Hair cortisol measurement may be a new diagnostic tool in clinical practice. Further research on HCC may contribute to improvement of the diagnosis and treatment of patients with HPA axis diseases. Hair cortisol concentration measurement, unlike other endocrinological tests, provides information about cortisol concentration in the patient's long-term history.

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