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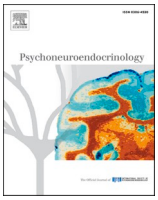

Long-term cortisol levels in hair of children and adolescents with Prader-Willi Syndrome

L. Damen [a,b,c,*], S.H. Donze [a,b,c], L.N. Grootjen [a,b,c], A.C.S. Hokken-Koelega [a,b,c]

[a] Dutch Growth Research Foundation, Rotterdam, The Netherlands
[b] Department of Pediatrics, Subdivision of Endocrinology Erasmus University Medical Center-Sophia Children's Hospital, Rotterdam, The Netherlands
[c] Dutch Reference Center for Prader-Willi Syndrome, The Netherlands

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ABSTRACT

Context: Prader-Willi syndrome (PWS) is characterized by hypothalamic dysfunction. In children with PWS, stress-induced central adrenal insufficiency (CAI) has been described, however, daily life cortisol production may be normal. Hair cortisol concentration (HCC) is a marker of long-term systemic cortisol production. Cortisol awakening response (CAR) is the increase in cortisol level after awakening. A negative CAR might suggest hypothalamic-pituitary-adrenal (HPA)-axis reactivity problems. Little is known about HCC and CAR in children with PWS.
Objective: To investigate long-term cortisol levels in hair and CAR in children with PWS.
Design: Cross-sectional study.
Patients: 41 children with PWS.
Setting: Dutch PWS Reference Center.
Main outcome measures: HCC and salivary cortisol measured by LCMS.
Results: Median (IQR) HCC was 1.90 (1.02–3.30) pg/mg at a median (IQR) age of 14.5 (8.20–19.0) years, with median HCC in age-matched references being 2.63 pg/mg. Five patients (13.2%) had HCC < 2.5th percentile for age and these patients had a repeatedly negative CAR. Median HCC was significantly lower in patients with negative CAR than in patients with normal CAR (1.00 (0.22–1.59) vs. 2.25 (1.47–3.26) pg/mg, $p = 0.007$). One patient had both HCC < 2.5th percentile and repeatedly low morning salivary cortisol levels and negative CAR, and was diagnosed with adrenal insufficiency by overnight metyrapone test.
Conclusions: HCC were normal in the majority of children with PWS. Our data suggest that children with HCC < 2.5th percentile and (repeatedly) negative CAR might possibly have adrenal insufficiency or delayed HPA-axis responsiveness.

1. Introduction

Prader-Willi syndrome (PWS) is a rare genetic disorder caused by the lack of expression of paternally inherited imprinted genes on chromosome 15q11-q13, due to a paternal deletion, maternal uniparental disomy (mUPD), imprinting center defect (ICD) or translocation (Goldstone et al., 2008; Cassidy and Driscoll, 2009). PWS is characterized by muscular hypotonia, abnormal body composition, developmental delay, behavioral problems, hyperphagia and endocrinopathies like growth hormone deficiency, hypothyroidism and hypogonadism (Holm et al., 1993; Cassidy, 1997; Eiholzer et al., 2000; Goldstone et al., 2008). Studies in children have reported a prevalence of central adrenal insufficiency (CAI) during stress in 0–60% of children with PWS (de Lind van Wijngaarden et al., 2008; Corrias et al., 2012; Obrynba et al., 2018; Oto et al., 2018).

Cortisol is produced by the adrenal cortex under the influence of (pituitary) adrenocorticotropic hormone (ACTH). There are different ways to assess cortisol levels. Cortisol can be measured in serum, saliva, urine and hair. A disadvantage of serum and saliva measurements is that they only provide a measurement of the cortisol concentration at a single point in time and are subject to major physiological circadian fluctuations, with a peak plasma cortisol level in the early morning (in healthy individuals) and a gradual decrease thereafter.

Nowadays, cortisol levels can be measured in hair samples (Sauve

* Correspondence to: Dutch Growth Research Foundation, Westzeedijk 106, 3016 AH Rotterdam, The Netherlands.
E-mail address: l.damen@kindengroei.nl (L. Damen).

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et al., 2007). Hair has a growth rate of approximately 1 cm/month. The last month's cortisol production is, therefore, represented by the most proximal 1 cm segment to the scalp (Sauve et al., 2007; Stalder and Kirschbaum, 2012). It is, therefore, possible to retrospectively examine cortisol production during a period of several months, by-passing the problem of daily circadian fluctuations. Recent studies have shown that hair cortisol is a marker of long-term systemic cortisol production (Russell et al., 2012; Stalder and Kirschbaum, 2012).

During stress conditions, like physical illness, surgery or major psychological stress, CAI has been reported in children with PWS (de Lind van Wijngaarden et al., 2008, Corrias et al., 2012, Obrynba et al., 2018). The reported prevalence of CAI varies, possibly as a result of different tests used (e.g. ACTH test or overnight metyrapone test (OMT)) (de Lind van Wijngaarden et al., 2008; Corrias et al., 2012; Obrynba et al., 2018). Our previous study in children with PWS showed normal morning salivary cortisol levels and diurnal profiles in all patients, also in the patients with stress-related CAI based on OMT (de Lind van Wijngaarden et al., 2008). Very recently, Shukur et al. (2020) described HCC in 29 adults with PWS and found that mean HCC was significantly higher in PWS adults than in healthy controls. However, HCC in children with PWS has never been investigated.

The cortisol awakening response (CAR) is the sharp increase in cortisol level 30–45 min after awakening, and is thought to be a measure of the reactive capacity of the hypothalamic-pituitary-adrenal axis (HPA-axis). In healthy adults, an increase of 50–150% in cortisol is considered as a normal CAR, while in children any increase is considered as normal (Clow et al., 2004; Rosmalen et al., 2005; Fries et al., 2009; Hadwin et al., 2019). Little is known about the CAR in patients with PWS.

In the current study, we analyzed cortisol production during 3 months in children and adolescents with PWS by measuring HCC. We hypothesized that daily cortisol production would be in the normal range. Furthermore, we investigated diurnal salivary cortisol and CAR, and hypothesized that these would also be normal. We subsequently investigated the relation between HCC and CAR.

2. Methods

2.1. Patients

Patients visiting the Dutch PWS Reference Center were asked to participate in the current evaluation of cortisol measurements in saliva and hair. Inclusion criteria were: (1) genetically confirmed diagnosis of PWS; (2) age 5–27 years; (3) receiving GH treatment for at least 1 year. A total of 41 children and adolescents were included. Parents of participants were asked to fill-out a questionnaire on hair care characteristics, use of glucocorticoid medication and stressful events during the past 3 months. Parents were also asked to collect saliva of their child during one day in each of the three consecutive months before collection of the hair sample at our outpatient clinic.

All patients had a hydrocortisone stress schedule in case of a serious illness or major psychological stress, according to Dutch guideline, unless a recent overnight metyrapone test (OMT) had ruled-out stress-related CAI.

This study was approved by the Medical Ethics Committee of the Erasmus University Medical Center, Rotterdam. Written informed consent was obtained from parents and participants older than 12 years of age.

2.2. Measurements

Patients were examined at the outpatient clinic of our PWS Reference Center and a hair sample was taken. Standing height was measured with a calibrated Harpenden stadiometer, and weight was determined on a calibrated scale (Servo Balance). Height, weight and body mass index (BMI) standard deviation scores (SDS) were calculated with Growth Analyser 4.0 (available at www.growthanalyser.org), and were adjusted for gender and age according to Dutch reference values (Fredriks et al., 2000a, 2000b). DXA (Lunar Prodigy; GE Healthcare) was used to measure percentage body fat. All scans were made on the same machine, and daily quality assurance was performed. Fasting blood samples were collected after an overnight fast to determine serum IGF-I levels and metabolic health parameters (glucose, insulin, cholesterol). All determinations in blood, hair and salivary samples were performed in the Biochemical and Endocrine laboratories of the Erasmus University Medical Center, Rotterdam.

2.3. Hair sample collection and analysis

Hair was cut from the posterior vertex, because this region shows the lowest intra-individual variation (Sauve et al., 2007), as close to the scalp as possible using small surgical scissors. The hair sample was taped to a paper form and stored in an envelope at room temperature until analysis. Hair samples were analyzed by the Clinical Chemistry Laboratory of the Erasmus University Medical Center, Rotterdam. The samples were processed and analyzed as described (Noppe et al., 2015; de Kruijff et al., 2020). For the current study the three most proximal centimetres were used. After solid phase extraction, hair cortisol was quantified per milligram of hair by liquid chromatography-tandem mass spectrometry (LCMS) using a Xevo TQ-S system (Waters Chromatography, Milford, MA) (de Kruijff et al., 2020).

Hair cortisol concentrations (HCC) were compared to age-matched reference values from a healthy study population, established by the same laboratory which performed the HCC analysis in the current study (Erasmus University Medical Center), using the exact same LCMS method (de Kruijff et al., 2020).

2.4. Diurnal cortisol profile

Salivary cortisol was collected at four time-points during the day: at wake-up, 30-min after wake-up, in the afternoon and before sleep. Parents were given detailed written instructions for collection (e.g. no food, drinks or brushing of the teeth in the 30 min before collection). Saliva was collected in Salivette tubes. Maximal morning salivary cortisol levels were defined as the highest cortisol level before 0800 h in the morning (either at wake-up or 30-min thereafter). Salivary cortisol samples were measured by LC-MS/MS. Reference ranges reported by the laboratory for morning salivary cortisol levels are 1.6–19.3 nmol/l. Cortisol awakening response (CAR) was categorized into a positive CAR (increase in cortisol after awakening) or negative CAR (no increase or a decrease in cortisol after awakening). As not all patients collected all 3 profiles, only the first collected profile was used to assess CAR.

2.5. Statistics

Statistical analyses were performed with SPPS version 24.0 (SPSS Inc., Chicago, IL). Variables are expressed as median (interquartile range [IQR]). HCC was log-transformed to achieve normal distribution. Independent sample *t*-test was used to investigate differences in HCC between groups. Spearman's correlation was used to investigate correlation of HCC with sex, age, BMI SDS, FM% SDS, glucose, insulin and serum IGF-I SDS. Friedman test was used to investigate if there was a significant difference between the 3 saliva profiles. Fisher's exact test was used to investigate if the proportion of patients with a negative CAR was different between patients with a normal HCC and those with a low HCC. Differences were considered significant if p-value was < 0.05.

3. Results

3.1. Clinical characteristics

Forty-one patients (20 females) had a hair sample taken for analysis

Table 1
Clinical characteristics at sampling of hair.

	Total group
Number (females)	41 (20)
Genetic subtype	
Deletion / mUPD	24 / 17
Age (yrs)	14.5 (8.2–19.0)
Height (SDS)	-0.32 (−1.5 to 0.6)
BMI for age (SDS)	1.1 (0.0–1.6)
Fat mass percentage (SDS)[a]	2.2 (1.9–2.6)
Fat mass percentage (%)	37.3 (32.1–42.4)
Lean body mass (SDS)[a]	-1.8 (−2.4 to −0.9)
GH dose (mg/m^2/day)	0.7 (0.6–1.0)
IGF-I SDS[a]	1.9 (1.2–2.9)

Data expressed as median (IQR). mUPD: maternal uniparental disomy. ICD: imprinting center defect. GH: growth hormone.

[a] FM% SDS, LBM SDS and IGF-I SDS were calculated according to age- and sex-matched Dutch references.

(Table 1). In one patient, hair cortisol measurement was not possible due to a technical error. Twenty-four patients had a deletion (58.5%) and seventeen (41.5%) a mUPD. Median (IQR) age was 14.5 (8.2–19.0) years and median BMI for age was 1.1 (0.0–1.6) SDS.

3.2. Hair cortisol concentration in children and adolescents with PWS

Fig. 1 shows the individual HCC compared to reference values for healthy children (de Kruijff et al., 2020). Median (IQR) HCC in the total group with PWS was 1.90 (1.02–3.30) pg/mg (normal reference range at age of 14 years: 2.5th percentile 0.47 pg/mg, median 2.63 pg/mg, 97.5th percentile: 15.5 pg/mg (de Kruijff et al., 2020)).

Five (13.2%) patients had an HCC below the 2.5th percentile for age (de Kruijff et al., 2020). These were all females and aged between 6 and 21 years. None had undergone hair treatment (e.g. dyeing, bleaching, or perming) or had used a hair product (e.g. wax, spray, mousse) on the day of sampling. One patient with HCC < 2.5th percentile underwent an OMT, which showed an 8 AM ACTH of 14.1 pmol/l and 11-DOC of 114.8 nmol/l. Another patient received occasionally 5 mg prednisolone a day by her mother due to vague complaints of fatigue, nausea and temperature swings. An OMT was performed in this last patient after the study period and showed an insufficient ACTH and 11-DOC response (8.8 pmol/l and 87.2 nmol/l resp.) with extremely low cortisol levels for which hydrocortisone substitution therapy was prescribed. The parents of the other 3 patients did not want an OMT.

None of the patients had an HCC above the 97.5th percentile for age (de Kruijff et al., 2020).

3.3. Hair cortisol concentration in various subgroups

There was no difference in HCC in patients aged < 18 years compared to those of 18 years and older (p = 0.53) (Table 2). HCC was lower, albeit not significant, in females than in males, with median hair cortisol concentration of 1.50 (0.30–3.70) pg/mg in females and 2.00 (1.20–3.00) pg/mg in males (p = 0.11). Seven males used an adult dose testosterone replacement therapy. There was no difference in median HCC between the genetic subtypes, nor between patients who did not use or did occasionally use oral hydrocortisone, due to illness according to stress schedule (varying between one dose of oral hydrocortisone to a maximum use of oral hydrocortisone for one week during a single illness episode), in the 3 months before hair sampling. The use of hair products (e.g. gel, wax or spray) on the day of hair collection did not result in a difference in median HCC.

HCC was inversely correlated with FM% SDS, (rho = −0.373, p = 0.02), but there was neither a correlation between HCC and BMI SDS or age, nor between HCC and serum IGF-I SDS, fasting serum glucose, insulin or cholesterol levels.

3.4. Morning salivary cortisol

A total of 67 wake-up salivary cortisol samples in 26 patients were collected before 0800 h. Three different patients had an early morning salivary cortisol below the reference range of 1.6 nmol/l, one of them during all 3 diurnal profiles collected on different days. This patient also had an HCC < 2.5th percentile for age. Another patient had a wake-up salivary cortisol level < 1.0 nmol/l at the first profile, but a normal 30-min after wake-up level of 1.8 nmol/l (also before 0800 h). This patient had a wake-up level of 5.6 nmol/l at the second profile and 5.7 nmol/l at the third profile. Unfortunately, measurement of HCC in this patient failed due to a technical error. The third patient had a

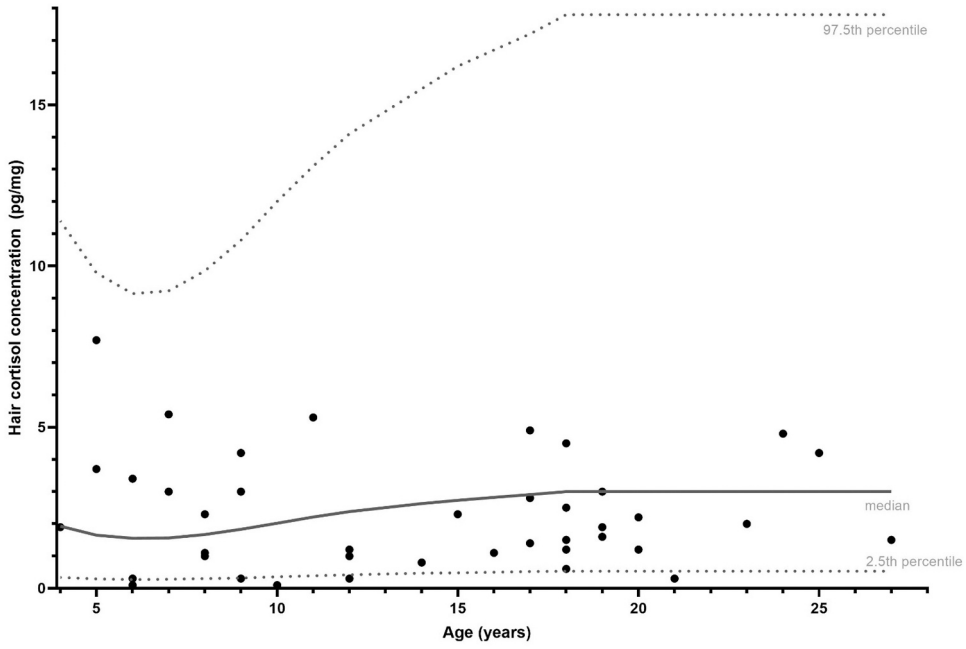

Fig. 1. Hair cortisol concentration in 40 children and adolescents with PWS. Reference range (2.5th, 50th and 97.5th) for healthy children according to age are depticited by the gray lines (24). Five patients have a hair cortisol concentration below the 2.5th percentile for age.

Table 2
Hair cortisol concentration in children with PWS.

	Hair cortisol concentration	*p-value
Total group	1.90 (1.02–3.3)	
Age		0.53
< 18 years (N = 25)	1.90 (0.89–3.55)	
≥ 18 years (N = 15)	1.90 (1.20–3.00)	
Sex		0.11
Females (N = 19)	1.50 (0.30–3.70)	
Males (N = 21)	2.00 (1.20–3.00)	
Genetic subtype		0.36
Deletion (N = 24)	1.73 (0.85–3.00)	
mUPD (N = 16)	1.90 (1.20–4.07)	
Incidental oral hydrocortisone use during stress 3 mo before collection		0.35
Yes (N = 11)	2.50 (1.10–3.40)	
No (N = 29)	1.60 (0.33–3.33)	
Use of any glucocorticoids* 3 mo before collection		0.27
Yes (N = 13)	2.20 (1.14–3.94)	
No (N = 17)	1.90 (0.89–2.54)	
Stressful events 3 mo before collection		0.46
Yes (N = 14)	2.57 (1.02–4.69)	
No (N = 21)	1.90 (0.77–0.32)	
Use of hair products (e.g. gel, wax, spray) on day of hair collection		0.38
Yes (N = 11)	1.90 (1.00–4.80)	
No (N = 25)	2.00 (0.49–3.19)	

Data expressed as median (IQR). # genetic subtype unknown. mUPD: maternal uniparental disomy. ICD: imprinting center defect.
GH: growth hormone. Hair products: wax, gel.
*Glucocorticoids used during the study: oral hydrocortisone (N = 11), inhalation steroids (N = 3), topical steroids on skin (not scalp) (N = 2).

normal wake-up salivary cortisol level of 1.8 nmol/l at the first profile, but a low wake-up level of 1.0 nmol/l and a 30-min after wake-up level < 1.0 nmol/l at the second profile, but did not collect a third profile. HCC was 2.2 pg/mg, which was normal for his age (2.5th percentile 0.53 pg/mg, median 3.00 pg/mg).

3.5. Diurnal cortisol profiles and CAR

Table 3 shows the diurnal cortisol profiles. Median (IQR) salivary cortisol level at wake-up during the first diurnal profile was 5.0 (3.4–8.5) nmol/l, and at 30-min after wake-up 5.9 (4.1–8.6) nmol/l. Median (IQR) salivary cortisol levels were lower in the afternoon and before sleep (1.4 (0.5–2.4) nmol/l and 1.0 (0.5–1.7) nmol/l, resp.).

Nineteen (65.5%) patients had an increase in cortisol levels after awakening (positive CAR), while 10 (34.5%) patients did not have an increase in cortisol levels after awakening (negative CAR). One patient had disturbed sleep and this patient had a negative CAR.

Median (IQR) HCC was 1.00 (0.22–1.59) pg/mg in patients with a negative CAR, which was significantly lower than the median (IQR) HCC of 2.25 (1.47–3.26) pg/mg in patients with a positive CAR (p = 0.007). Table 4 shows the contingency table for all patients with both HCC and CAR measurements. The proportion of patients with a negative CAR was significantly higher in patients with a low HCC than in those with a normal HCC (p = 0.010). Of the 5 patients with HCC below the 2.5th percentile for age, 4 patients had 3 diurnal profiles and a negative CAR in all 3 profiles. Only two of these patients underwent an OMT which showed an insufficient ACTH response.

4. Discussion

This study in 41 children and adolescents with PWS shows that HCC is normal in the majority (86.8%) of children and adolescents with PWS, which implies a normal cortisol production during daily life. HCC, in combination with negative CAR and low early morning salivary cortisol,

Table 3
Cortisol in saliva.

Cortisol in saliva					
Salivary cortisol at wake-up (nmol/l)	7.5 (4.4–11.0)				
Salivary cortisol 30 min after wake-up (nmol/l)	7.9 (5.4–9.9)				
Salivary cortisol in the afternoon (nmol/l)	1.6 (0.9–2.5)				
Salivary cortisol before asleep (nmol/l)	1.2 (0.5–2.4)				
Variables are expressed as median (IQR). Average of 3 measurements on 3 different days.					
Cortisol in saliva					
Profile (N = 33)					
Salivary cortisol at wake-up (nmol/l)	5.0 (3.4–8.5)				
Salivary cortisol 30 min after wake-up (nmol/l)	5.9 (4.1–8.6)				
Salivary cortisol in the afternoon (nmol/l)	1.4 (0.5–2.4)				
Salivary cortisol before asleep (nmol/l)	1.0 (0.5–1.7)				
Positive CAR (number (%))	19 (65.5%)				
Negative CAR (number (%))	10 (34.5%)				
Cortisol in saliva in patients who collected all 3 profiles (N = 18)					
Profile	*Profile 1*	*Profile 2*	*Profile 3*	**p-value*	*reference range*
Salivary cortisol at wake-up (nmol/l)	6.3 (3.8–8.9)	6.2 (3.9–9.2)	5.8 (4.0–7.8)	0.65	1.6–19.3
Salivary cortisol 30 min after wake-up (nmol/l)	6.0 (3.3–8.3)	7.6 (4.6–10.3)	5.0 (3.5–7.3)	0.066	1.6–19.3
Salivary cortisol in the afternoon (nmol/l)	1.4 (0.5–2.4)	1.4 (0.5–2.6)	0.9 (0.5–2.5)	0.68	
Salivary cortisol before asleep (nmol/l)	1.1 (0.5–1.7)	0.5 (0.5–1.5)	0.5 (0.5–1.5)	0.62	

Variables are expressed as median (IQR).
CAR: cortisol awakening response. Positive CAR: an increase in cortisol levels after wake-up. Negative CAR: no increase in cortisol levels after wake-up.
A positive CAR is considered normal.
* p-value: the p-value of the difference between profile 1, profile 2 and profile 3 calculated with Friedman test.

Table 4
Contingency table for patients with both HCC and CAR measurements.

	CAR -	CAR +	Total
Normal HCC	6	18	24
Low HCC[a]	4	0	4
Total	10	18	28

CAR: cortisol awakening response.
HCC: hair cortisol concentration.
p-value: 0.010.
[a] Low HCC was defined as HCC < 2.5th percentile.

was able to identify the one patient with insufficient cortisol production. Five patients had a HCC < 2.5th percentile, four of them had a (repeatedly) negative CAR (one did not have CAR determination), and the two patients who underwent an OMT had an insufficient ACTH and 11-DOC response. As CAR has been described as measure of reactive capacity of the HPA-axis, our data suggest that children with an HCC < 2.5th percentile and (repeatedly) negative CAR might possibly have adrenal insufficiency or delayed HPA-axis responsiveness.

The normal HCC in most children and adolescents with PWS are in contrast with a recent study showing an elevated mean HCC in 29 adults with PWS (mean age 33.4 years) compared to healthy controls, with HCC measurements performed in the same laboratory as the current study (Shukur et al., 2020). In that study, mean HCC was 12.8 pg/mg in PWS and 3.8 pg/mg in healthy controls, while in our study median HCC was 1.90 pg/mg at a median age of 14.5 years compared to a median HCC of 2.63 pg/mg in healthy children aged 14 years (de Kruijff et al., 2020). However, that study had 2 very high readings of HCC and exclusion resulted in a mean HCC of 6.4 pg/mg. A possible explanation for the higher HCC in that study could be, that there is a lower daily cortisol production in children than in adults with PWS. A functional difference in cortisol production between children and adults is also suggested by the different results found regarding stress-related CAI, where in children a higher prevalence is reported (de Lind van Wijngaarden et al., 2008; Rosenberg et al., 2020). Furthermore, 20.0% of patients in the study by Shukur et al. were overweight and 41.4% obese, while in our study only 5 (12.2%) were obese with a BMI > 2 SDS for age. Although we did not find an association between BMI and HCC, the study by Shukur et al. did, and therefore, the higher BMI could also play a role in the higher HCC.

Surprisingly, we found an inverse correlation between HCC and FM% SDS in children and adolescents with PWS. This is in contrast with other studies in participants without PWS showing a positive correlation between HCC and body fat or fat mass index (Larsen et al., 2016; Noppe et al., 2016; Gerber et al., 2017). Multiple studies have also described a positive correlation between HCC and BMI or obesity (Chan et al., 2014; Wester et al., 2014; Wagner et al., 2019; Ling et al., 2020; Shukur et al., 2020), while we did not find a correlation between HCC and BMI SD-scores. However, our findings are in accordance with few other studies (Wester et al., 2017; Genitsaridi et al., 2019). As the majority of our patients had a BMI SD-score in the normal range, it is possible that the correlation becomes only significant when the BMI range is larger because more obese patients are included. The fact that we found an inverse correlation between HCC and FM% SDS might be due to the abnormal body composition in all patients with PWS. It might be that the correlation is reversed when all patients are in the in the upper range of normal fat mass.

We neither found a difference in HCC between patients younger or older than 18 years, nor between genetic subtype. However, the majority of patients older than 18 years were only aged 18–21 years. It might be that differences between children and adults with PWS become only apparent at an older age. We found a higher HCC in males than in females, albeit not significant, which is in accordance with studies in healthy populations reporting a higher HCC in men (Rippe et al., 2016; Gerber et al., 2017, Wester et al., 2017; Wagner et al., 2019). We did not find a significant difference between patients who used or did not use a hair product on the day of collection, which is in accordance with other studies (Wester et al., 2017; de Kruijff et al., 2020).

Eleven patients used occasionally oral hydrocortisone for 1 or 2 days during acute stress in the 3-month period prior to hair sampling. There was no difference in HCC between patients who used hydrocortisone and those who did not. Other studies have shown that HCC is elevated in patients on hydrocortisone substitution therapy for AI and stated that HCC could be useful for monitoring hydrocortisone substitution therapy (Gow et al., 2011; Manenschijn et al., 2011; Noppe et al., 2014). The fact that we did not find an elevated HCC in children and adolescents who used hydrocortisone once or twice a month during illness or psychological stress, according to Dutch guideline, is reassuring and suggests that there is no overtreatment of hydrocortisone in these patients.

Measuring HCC has a number of advantages compared to other methods to estimate daily cortisol production. Hair sampling is non-invasive, easy and quick, not subject to daily fluctuations and does not depend on adherence of a patient in collecting salivary samples for diurnal profiles. We suggest that HCC can be used as a non-invasive screening tool for insufficient cortisol production in daily life. If a low HCC is found, additional tests should be performed.

In addition to HCC, we investigated diurnal salivary cortisol profiles. Almost all children and adolescents had normal morning salivary cortisol levels which confirms normal daily cortisol production in most children with PWS (de Lind van Wijngaarden et al., 2008). However, we found that children and adolescents with a negative CAR had a significantly lower HCC than those with a positive CAR and that the proportion of patients with a negative CAR was significantly higher in the patients with a low HCC than in those with normal HCC. As CAR has been described to be a measure of reactive capacity of the HPA-axis (Schmidt-Reinwald et al., 1999), a negative CAR might suggest a delayed responsiveness of the HPA-axis during acute stress which might support the presence of stress-related CAI. However, in a study in healthy children, 30% had a negative CAR (Rosmalen et al., 2005). Therefore, the predictive value of a negative CAR only is low. The combination of a low HCC and a negative CAR might be better able to identify patients with HPA-axis problems than CAR alone. Future studies should be performed to confirm this finding. A delayed or insufficient responsiveness during acute stress was also found during an insulin tolerance test or OMT in children with PWS (de Lind van Wijngaarden et al., 2008; Oto et al., 2018).

Five (13.2%) patients had an HCC far below the 2.5th percentile for age. Three of these patients had normal morning salivary cortisol levels and one patient had morning levels below the reference range, but all had a negative CAR on all 3 salivary profiles and two of them underwent an OMT showing an insufficient ACTH response. Unfortunately, parents of the other three patients did not want an OMT. The patients with normal morning salivary cortisol levels did not experience symptoms of AI in daily life, but might have less HPA-axis reactivity given the combination of HCC < 2.5th percentile and negative CAR. Future studies should be performed to confirm the relation between HCC, CAR and delayed responsiveness of the HPA-axis. If our findings are confirmed, we would suggest to perform an OMT in children with a low HCC and/or (repeatedly) negative CAR to exclude stress-related CAI or administer hydrocortisone in stress dose during periods of acute stress. Until then, we advise to administer hydrocortisone in stress dose during acute stress in all children with PWS, unless stress-related CAI is ruled-out by an OMT (de Lind van Wijngaarden et al., 2008).

One patient had an HCC far below the 2.5th percentile for age, repeatedly low morning salivary cortisol levels, repeatedly negative CARs and, after the present study, an insufficient ACTH response and early morning low 11-DOC level was found during an OMT. This indicates that HCC could be used as a screening tool to identify a patient at risk for insufficient daily cortisol production. This patient was occasionally given 5 mg of prednisolone (not according to Dutch guideline) a day by her mother during the study period due to vague complaints of fatigue, nausea and temperature swings. Since prednisolone is not determined by LCMS, we cannot differentiate if this patient had these complaints due to CAI or because she had been receiving more prednisolone than reported and consequently developed iatrogenic AI with low endogenous cortisol production as a result.

A limitation of the current study is the relatively small population, although the sample size is large for a rare disorder like PWS. We did not have a control group in this study, but used the reference ranges for HCC established in the same laboratory where current hair samples were analyzed under the same conditions (de Kruijff et al., 2020). We were, therefore, able to adequately interpret HCC as normal or abnormal in

comparison to age-matched peers.

In conclusion, this is the first study investigating long-term cortisol production measured by HCC in children and adolescents with PWS. Current data help to better acknowledge the wide spectrum of HPA-axis reactivity occurring in PWS children. We show that cortisol production was normal in the majority of children and adolescents. The one patient with insufficient cortisol production could be identified via the combination of low HCC, a negative CAR and low early morning salivary cortisol. All children and adolescents with low HCC and CAR determination had a negative CAR on multiple salivary cortisol profiles. Median HCC was lower in children and adolescents with a negative CAR than in those with a normal CAR. Although cortisol production in daily life is normal, cortisol production during acute stress can still be abnormal in children with PWS (de Lind van Wijngaarden et al., 2008). The two patients with low HCC and negative CAR who underwent an OMT, showed insufficient ACTH response. Thus, HCC and a (repeatedly) negative CAR might possibly be able to identify children with adrenal insufficiency or a delayed responsiveness of the HPA-axis during acute stress, but this needs further research. The current study also shows that the occasional use of hydrocortisone for stress-related CAI does not result in hydrocortisone overtreatment.

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Data Availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Conflict of interest statement

None of the authors of the manuscript *"Long-term cortisol levels in hair of children and adolescents with Prader-Willi Syndrome"* have any conflict of interest to declare.

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