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## RESEARCH

# Sexual dimorphism in cortisol metabolism throughout pubertal development: a longitudinal study

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## Abstract

**Objective:** Sex differences in disease susceptibility might be explained by sexual dimorphism in hypothalamic-pituitary-adrenal axis activity, which has been postulated to emerge during puberty. However, studies conducted thus far lacked an assessment of Tanner pubertal stage. This study aimed to assess the contribution of pubertal development to sexual dimorphism in cortisol production and metabolism.

**Methods:** Participants ( $n = 218$ ) were enrolled from a population-based Netherlands Twin Register. At the ages of 9, 12 and 17 years, Tanner pubertal stage was assessed and early morning urine samples were collected. Cortisol metabolites were measured with GC-MS/MS and ratios were calculated, representing cortisol metabolism enzyme activities, such as A-ring reductases, 11 $\beta$ -HSDs and CYP3A4. Cortisol production and metabolism parameters were compared between sexes for pre-pubertal (Tanner stage 1), early pubertal (Tanner stage 2–3) and late-pubertal (Tanner stage 4–5) stages.

**Results:** Cortisol metabolite excretion rate decreased with pubertal maturation in both sexes, but did not significantly differ between sexes at any pubertal stage, although in girls a considerable decrease was observed between early and late-pubertal stage ( $P < 0.001$ ). A-ring reductase activity was similar between sexes at pre- and early pubertal stages and was lower in girls than in boys at late-pubertal stage. Activities of 11 $\beta$ -HSDs were similar between sexes at pre-pubertal stage and favored cortisone in girls at early and late-pubertal stages. Cytochrome P450 3A4 activity did not differ between sexes.

**Conclusions:** Prepubertally, sexes were similar in cortisol parameters. During puberty, as compared to boys, in girls the activities of A-ring reductases declined and the balance between 11 $\beta$ -HSDs progressively favored cortisone. In addition, girls showed a considerable decrease in cortisol metabolite excretion rate between early and late-pubertal stages. Our findings suggest that the sexual dimorphism in cortisol may either be explained by rising concentrations of sex steroids or by puberty-induced changes in body composition.

## Key Words

- ▶ glucocorticoid
- ▶ metabolites
- ▶ steroid
- ▶ Tanner
- ▶ sex differences

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## Introduction

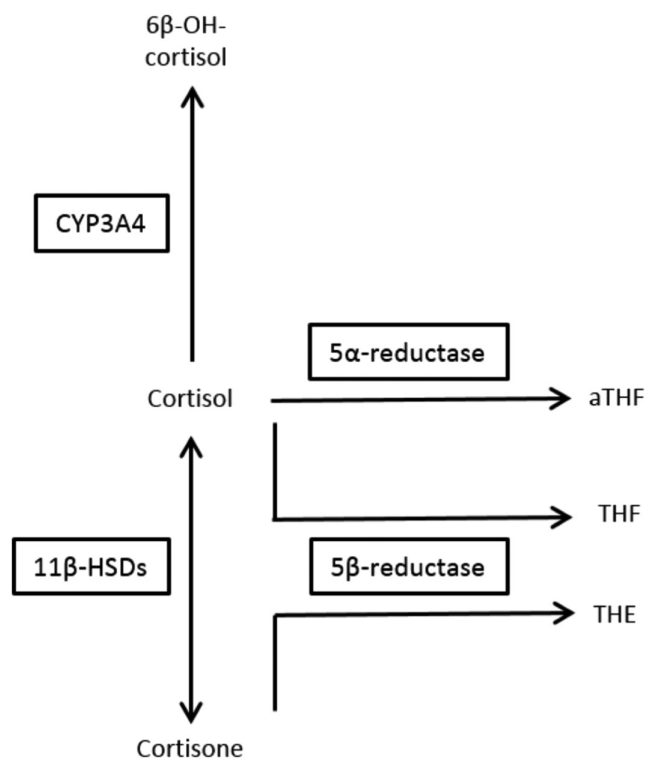
Males and females differ in their susceptibility to develop specific diseases. While females are more likely to develop auto-immune diseases and neuropsychiatric disorders like anxiety and depression, males are more susceptible to infectious diseases and are more likely to engage in violent competition (1, 2, 3). Moreover, males and females differ in cardiovascular disease susceptibility (4). Sex differences in HPA-axis settings have been hypothesized to play a role in these differences (5, 6, 7, 8, 9, 10).

Sexual dimorphism in HPA-axis activity has been suggested to be already present in early childhood. A recent meta-analysis suggested that boys and girls differed in basal HPA-axis activity, as assessed by salivary cortisol levels. Compared to girls, boys, up to age 8, had higher salivary cortisol levels and lower levels beyond this age (11). The timing of this change suggests that sex steroids influence the HPA axis. Surprisingly, to the best of our knowledge, there are no studies that have reported on HPA-axis activity across pubertal development.

HPA-axis activity is determined by the net effect of cortisol production and metabolism. Cortisol is metabolized by various enzymes (Fig. 1). The A-ring reductases ( $5\alpha$ - and  $5\beta$ -reductase), together with cytochrome P (CYP) 3A4, eliminate cortisol from the circulation primarily in the liver (12). The 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) isozymes regulate the interconversion between cortisol and its inactive metabolite cortisone. 11 $\beta$ -HSD type 1 is mainly expressed in the liver and adipose tissue, where it regenerates cortisol, and 11 $\beta$ -HSD type 2 catalyzes the reverse reaction in renal epithelial cells.

In adulthood, females were found to have a lower urinary excretion rate of cortisol metabolites than males (13, 14, 15). This is likely to be attributed to less A-ring reduction in females, resulting in a prolonged half-life of cortisol and, hence, enhanced central feedback suppression (14). In contrast, CYP3A4 activity is higher in women than in men. However, CYP3A4 is known to eliminate only a small proportion of circulating cortisol (16, 17). There is controversy as to whether men and women differ in the activities of 11 $\beta$ -HSDs (14, 18, 19, 20).

Interaction between gonadal steroids and the metabolism of cortisol has been suggested by several studies (21, 22, 23, 24). However, there is only one cross-sectional study that has investigated glucocorticoid metabolism in children of various ages (18). This study demonstrated that the sex differences in the elimination rate of cortisol, as observed in adulthood, began around the age of



**Figure 1**

Schematic overview of cortisol metabolism. THF, tetrahydrocortisol; THE, tetrahydrocortisone; aTHF, allotetrahydrocortisol; 6 $\beta$ -OH-cortisol, 6 $\beta$ -hydroxy-cortisol; HSD, hydroxysteroid dehydrogenase; CYP, cytochrome P.

11–12 years and were attributable to a progressive difference in  $5\alpha$ -reductase activity (being lower in older girls). Therefore, sexual dimorphism in cortisol metabolism was postulated to emerge during pubertal maturation, suggesting an interplay of adrenal and gonadal axes (18). However, information on pubertal stage was not available in that study (18). To the best of our knowledge, a longitudinal follow-up study of cortisol metabolism from pre- to post-puberty has never been conducted. The aim of this study was to assess the contribution of pubertal development to sexual dimorphism in cortisol production and metabolism.

## Materials and methods

### Participants

We conducted a prospective follow-up study and recruited healthy mono- and dizygotic twin pairs from the Netherlands Twin Register (NTR), a population-based registry (25, 26). Twins born between 1995 and 1996 were invited to participate in the BrainScale study of cognition, hormones, and brain development (27, 28). BrainScale is a

collaborative project between the NTR of the VU University Amsterdam and the University Medical Center Utrecht. Four to 8 weeks before the ninth birthday of the twins, a letter of invitation was sent to their parents. Following the invitation letter, the families were contacted by phone to inquire whether they were willing to participate. At the age of 9 years, 109 of the invited twin pairs (51%) participated in this study. Eighty-two percent of them participated at the age of 12 years, and 80% participated at the age of 17 years. Seventy-one percent participated at all three occasions. Parents signed informed consent forms for their participating children and for themselves. In addition, twins signed their own informed consent forms at the third occasion (i.e. at the age of 17 years). The current study was approved by the medical ethics committee of the Amsterdam UMC, location VUmc.

Participants were physically examined by a researcher for pubertal stage at ages 9 and 12 years. The examination was based on secondary sexual characteristics according to Marshall and Tanner, that is, breast stage in girls and genital stage in boys (29, 30). Prior to the study, researchers were trained in the assessment of pubertal stage. If children felt uncomfortable with physical examination, pubertal stage was determined by self-report based on reference images. Of the participants, 100% and 80% were physically examined for pubertal stage at the ages of 9 and 12 years, respectively. At the age of 17 years, pubertal stage was always determined by self-report. In addition, at the age of 17 years, information on the timing of menarche was ascertained. Previous data showed that the inter-rater agreement of self-reported data is relatively low, ranging between 49 and 86% in girls and between 27 and 78% in boys, in comparison to researcher-reported data (31, 32, 33, 34, 35, 36, 37). Therefore, we classified participants as pre-pubertal (Tanner stage 1, hereafter referred to as stage A), early pubertal (Tanner stages 2 and 3, hereafter referred to as stage B), or late-pubertal (Tanner stages 4 and 5, hereafter referred to as stage C). Girls documenting menarche were classified as late-pubertal, regardless of

Tanner stage. For self-reported data, our classification system is expected to have an inter-rater agreement of 82% in girls and of 75% in boys, based on Danish reference data (31). These numbers are comparable with the inter-rater reliability of individual Tanner stages between trained assessors (38).

### Study protocol

Participants were examined at the ages of 9, 12, and 17 years. In the week prior to their study visit, subjects collected first voided morning urine samples in tubes provided by us. Participants were requested to store the samples in their refrigerator and to hand them in at the study visit. Samples were stored at  $-20^{\circ}\text{C}$  and then at  $-80^{\circ}\text{C}$ . Samples were thawed only once just before analysis.

### Laboratory analysis

The analysis of cortisol metabolites was conducted at the Edinburgh Clinical Research Facility Mass Spectrometry Core Laboratory. Glucocorticoid metabolites were measured by gas chromatography-tandem mass spectrometry (GC-MS/MS) (39). Samples were analyzed in 15 batches. Creatinine concentrations were measured by the Jaffé method (40). The sum of cortisol metabolites divided by creatinine concentration was used as an index of cortisol production (cortisol metabolite excretion rate). Enzymatic activities were inferred from cortisol metabolite ratios, as depicted in Table 1. Higher ratios indicate higher enzymatic activity, except for  $11\beta$ -HSD type 2 activity (cortisol/cortisone ratio).

### Statistical analysis

In line with previous analyses in this sample, extreme outliers ( $>3$  s.d. above the phenotypic mean or twin pairs with highly discordant outcomes; on average six and one per index, respectively) were excluded from the statistical

**Table 1** Summary of outcomes.

Parameter	Index
(THF + allo-THF + THE + $\alpha$ -cortol + $\beta$ -cortol + $\alpha$ -cortolone + $\beta$ -cortolone)/creatinine	Sum of cortisol metabolites (cortisol metabolite excretion rate)
allo-THF/F	5 $\alpha$ -reductase activity
THF/F	5 $\beta$ -reductase activity (a)
THE/E	5 $\beta$ -reductase activity (b)
F/E	11 $\beta$ -HSD type 2 activity
(THF + allo-THF)/THE	Balance of 11 $\beta$ -HSD activities
6 $\beta$ -OH cortisol/F	Cytochrome P450 3A4 activity

E, cortisone; F, cortisol; HSD, hydroxysteroid dehydrogenase; THE, tetrahydrocortisone; THF, tetrahydrocortisol.

analysis (41). Next, the data were corrected for batch effects by fitting a random effects model, in which batch was treated as a random effect (42).

### Statistical modeling

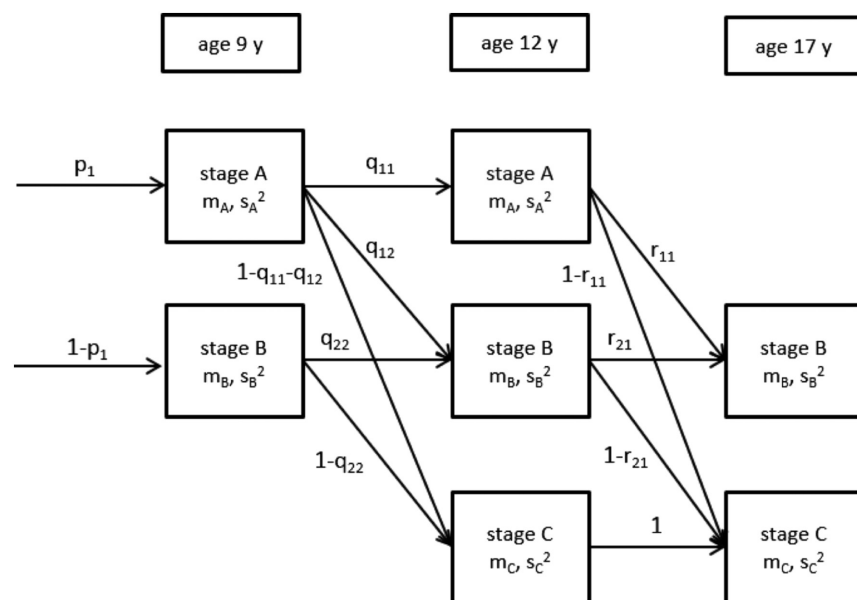
Our main aim was to determine the effect of pubertal stage on the outcomes. To this end, we implemented a discrete-time Markov model, in which we estimated the mean and s.d. of each metabolite conditional on the pubertal stage. In this model, each participant was assigned to stage A, B, or C. We took into account that a given participant may transition from stage A to B or C and from stage B to stage C between ages 9 and 12 and between 12 and 17 years, respectively. The parameters of the Markov model are the probabilities of being in stage A, B, or C at the age of 9 years, transition probabilities from ages 9 to 12 years, and transition probabilities from ages 12 to 17 years. Because no 9-year olds were assigned to stage C and no 17-year olds were assigned to stage A, the Markov model includes stages A and B at the age of 9 years, stages A, B, and C at the age of 12 years, and stages B and C at the age of 17 years. Figure 2 depicts the model. In Fig. 2,  $p_1$  is the probability of being in stage A at the age of 9 years,  $q_{11}$  and  $q_{12}$  are conditional probabilities governing the transition between ages 9 and 12 years, and  $r_{11}$  and  $r_{21}$  are conditional probabilities governing the transition between ages 12 and 17 years. For instance,  $q_{12}$  is the probability of transitioning from stage A at the age of 9 years to stage B at the age of 12 years.

As we expected sex differences in probability of the pubertal stages at the age of 9 years and in the transition probabilities from ages 9 to 12 years and from 12 to 17 years, we allowed these parameter to vary with sex (i.e. the probabilities  $p_1$ ,  $q_{11}$ ,  $q_{12}$ ,  $q_{22}$ ,  $r_{11}$ , and  $r_{21}$  varied with sex). In addition, we allowed for sex differences in the means of the metabolites within a given pubertal stage (i.e. the means  $m_A$ ,  $m_B$ , and  $m_C$  varied with sex). s.d. were constrained to be equal over sexes.

The Markov transition model was implemented in Mplus 6.0 (43). The parameters were estimated by means of maximum likelihood (ML) estimation. For this study, twins were treated as individuals. Therefore, the s.e., CIs, and test statistics were corrected for family clustering. For all outcomes, means were calculated for girls and boys along with their 95% CIs. Differences between sexes were tested and reported by a two-tailed  $P$  value, whereby a  $P$  value of  $< 0.05$  was considered as statically significant. Given the sample size, correction for multiple testing was not conducted. In addition, changes in cortisol parameters were calculated during pubertal development for both sexes.

### Results

A total of 218 participants (50% females) were included in this study, including 94 monozygotic and 124 dizygotic twins. The monozygotic twin pairs included 23 male and 24 female pairs. The dizygotic twin pairs included 22 male-, 21 female-, and 19 opposite-sex pairs. Participants were tested at 9.1 ( $\pm 0.1$ ), 12.2 ( $\pm 0.3$ ), and



**Figure 2** Statistical model: means depending on pubertal stage and sex. \*A = pre-pubertal (Tanner stage 1); B = early pubertal (Tanner stage 2–3); C = late-pubertal (Tanner stage 4–5).  $p_1$  is the probability of being in stage A at the age of 9 years,  $q_{11}$  and  $q_{12}$  are conditional probabilities governing the transition between ages 9 and 12 years, and  $r_{11}$  and  $r_{21}$  are conditional probabilities governing the transition between ages 12 and 17 years.

**Table 2** Characteristics of participants.

	Boys			Girls		
	9 years (n = 106)	12 years (n = 83)	17 years (n = 77)	9 years (n = 106)	12 years (n = 86)	17 years (n = 95)
Length						
cm	139.2 ± 5.5	151.8 ± 7.2	179.6 ± 6.2	138.2 ± 4.8	152.5 ± 7.2	168.6 ± 6.1
SDS	0.03 ± 0.89	-0.57 ± 0.93	-0.23 ± 0.83	0.06 ± 0.76	-0.60 ± 1.05	-0.10 ± 0.93
Weight						
kg	31.6 ± 4.3	42.5 ± 8.4	67.3 ± 9.4	31.3 ± 4.6	43.8 ± 8.4	61.3 ± 8.88
SDS	0.34 ± 0.95	0.38 ± 0.91	0.12 ± 1.03	0.29 ± 0.91	0.05 ± 2.91	0.37 ± 1.09
Body mass index						
kg/m <sup>2</sup>	16.2 ± 1.4	18.6 ± 2.0	20.9 ± 2.5	16.4 ± 2.0	18.8 ± 2.9	21.6 ± 3.3
SDS	0.21 ± 0.83	0.58 ± 0.86	0.21 ± 1.03	0.09 ± 1.00	0.32 ± 1.11	0.30 ± 1.13
Tanner stage (%) <sup>a</sup>						
A	94	24	0	81	10	0
B	6	70	20	19	62	0
C	0	5	80	0	28	100

Values represent mean ± s.d. or %.

<sup>a</sup>Percentage of patients in Tanner stages are based on the transition probabilities according our Markov model. A = pre-pubertal (Tanner stage 1); B = early pubertal (Tanner stage 2-3); C = late-pubertal (Tanner stage 4-5).

17.2 (±0.2) years of age. In total 542 samples were analyzed, of which 213 (50% females), 167 (50% females), and 162 (63% females) were obtained at the ages of 9, 12 and 17 years, respectively. Mean s.d. score (Z score) (±s.d.) BMI (weight(kg)/height(m)<sup>2</sup>) was 0.14 (± 0.93), 0.45 (± 1.00), and 0.27 (± 1.08) at the ages of 9, 12 and 17 years, respectively (44). **Table 2** displays the characteristics for boys and girls separately at the ages of 9, 12, and 17 years.

### Pubertal development

The Markov model parameter estimates are shown in **Table 3**. At the age of 9 years, as expected, boys were more likely to be pre-pubertal than girls (0.944 vs 0.811). At the age of 12 years, the entire spectrum of pubertal stages were observed in both sexes, although in girls puberty was generally more advanced. At the age of 17 years, all girls were classified as late-pubertal, while, contrary to expectation, still 20% of the boys were early pubertal.

Consequently, sex differences in the transition probabilities from 9 to 12 years and from 12 to 17 years were also observed. For instance, the probability of remaining in stage A between the ages of 9 and 12 years was markedly higher in boys (0.259) than in girls (0.127). The probability of moving from stage A to C between 9 and 12 years was higher in girls than in boys (0.282 vs 0.025).

### Sexual dimorphism in cortisol production and metabolism during pubertal development

**Table 4** displays the sex-specific means for cortisol metabolite excretion rate and cortisol metabolite ratios

by pubertal stage. Cortisol metabolite excretion rate did not statistically differ between sexes at any stage. The cortisol metabolite ratios were similar between sexes at stage A, but diverged during pubertal development. In boys at stages B and C, as compared to girls of the same stage, the balance of 11β-HSD activities ((THF+allo-THF)/THE ratio) favored cortisol. At stage B, this difference could be partially attributable to a lower 11β-HSD type 2 activity (cortisol/cortisone ratio) in boys. Girls at stage C, as compared to boys of the same stage, had lower activities of 5α- (allo-THF/F ratio) and 5β-reductases (THE/E ratio). There were no differences in CYP3A4 activity (6-OH cortisol/cortisol ratio) between sexes at stages B or C.

**Table 3** Pubertal development and transition probabilities according our Markov model.

Age (years)	Pubertal development <sup>a</sup>	Markov parameters	Girls	Boys
9	A	p <sub>1</sub>	0.811	0.944
	B	1-p <sub>1</sub>	0.189	0.056
9->12	A->A	q <sub>11</sub>	0.127	0.259
	A->B	q <sub>12</sub>	0.592	0.717
	A->C	1-q <sub>11</sub> -q <sub>12</sub>	0.282	0.025
	B->B	q <sub>22</sub>	0.733	0.500
	B->C	1-q <sub>22</sub>	0.267	0.500
12->17	A->B	r <sub>11</sub>	0.000	0.491
	A->C	1-r <sub>11</sub>	1.000	0.509
	B->B	r <sub>21</sub>	0.000	0.110
	B->C	1-r <sub>21</sub>	1.000	0.890
	C->C	1	1.000	1.000

<sup>a</sup>A = pre-pubertal (Tanner stage 1), B = early pubertal (Tanner stage 2-3), C = late-pubertal (Tanner stage 4-5).

**Table 4** Cortisol metabolite excretion rate and cortisol metabolite ratios by pubertal stage.

Parameter	Pubertal stage <sup>a</sup>	Mean girls (95% CI)	Mean boys (95% CI)	Variances	Sex difference	Effect size
					P value	Cohen's D
Cortisol metabolite excretion rate	A	0.581 (0.533–0.629)	0.571 (0.511–0.631)	0.047	0.777	0.046
	B	0.577 (0.522–0.631)	0.512 (0.446–0.579)	0.047	0.108	0.300
	C	0.448 (0.407–0.489)	0.487 (0.411–0.563)	0.056	0.400	0.165
5 $\alpha$ -reductase activity (allo-THF/F)	A	8.982 (8.087–9.876)	9.405 (8.158–10.653)	24.048	0.576	0.086
	B	9.909 (8.832–10.987)	8.255 (6.784–9.726)	21.623	0.064	0.356
	C	5.504 (4.724–6.284)	8.558 (7.135–9.981)	19.815	<b>&lt;0.001</b>	0.686
5 $\beta$ -reductase activity (a) (THF/F)	A	9.164 (8.388–9.939)	10.195 (9.014–11.377)	20.976	0.151	0.225
	B	11.116 (10.413–11.820)	11.367 (9.809–13.223)	29.161	0.791	0.046
	C	9.792 (9.096–10.487)	10.729 (9.453–12.006)	15.494	0.227	0.238
5 $\beta$ -reductase activity (b) (THF/E)	A	27.101 (25.358–28.845)	27.409 (25.140–29.678)	70.990	0.823	0.037
	B	31.517 (28.929–34.106)	29.465 (25.960–32.969)	132.215	0.335	0.178
	C	26.096 (24.404–27.787)	30.468 (26.861–34.076)	123.162	<b>0.046</b>	0.394
11 $\beta$ -HSD type 2 activity (F/E)	A	0.896 (0.846–0.946)	0.872 (0.799–0.945)	0.100	0.593	0.076
	B	0.772 (0.688–0.855)	0.915 (0.809–1.022)	0.106	<b>0.027</b>	0.439
	C	1.264 (1.168–1.359)	1.242 (1.102–1.382)	0.217	0.796	0.047
Balance of 11 $\beta$ -HSD activities ((THF + allo-THF)/THE)	A	0.613 (0.572–0.655)	0.623 (0.572–0.675)	0.035	0.741	0.053
	B	0.552 (0.497–0.607)	0.640 (0.570–0.711)	0.054	<b>0.040</b>	0.379
	C	0.669 (0.631–0.708)	0.793 (0.716–0.871)	0.056	<b>0.008</b>	0.524
Cytochrome P450 3A4 activity (6 $\beta$ -OH cortisol/F)	A	1.893 (1.748–2.038)	1.955 (1.734–2.176)	0.630	0.645	0.078
	B	1.907 (1.712–2.101)	1.834 (1.560–2.109)	0.910	0.663	0.077
	C	1.773 (1.606–1.940)	1.859 (1.598–2.120)	0.604	0.590	0.111

<sup>a</sup>A = pre-pubertal (Tanner stage 1); B = early pubertal (Tanner stage 2–3); C = late-pubertal (Tanner stage 4–5).

**Table 5** presents the changes in cortisol metabolite excretion rate and cortisol metabolite ratios during pubertal development by sex. In both sexes cortisol metabolite excretion rate decreased with pubertal progression, albeit with few differences. In boys, cortisol metabolite excretion rate decreased between stages A and B, while in girls it decreased considerably between stages B and C. In both sexes between stages B and C the balance of 11 $\beta$ -HSD activities ((THF + allo-THF)/THE) changed in the direction of cortisol, which could partially be explained by decreased 11 $\beta$ -HSD type 2 activity (cortisol/cortisone ratio). In girls, the activities of A-ring reductases (ratios of allo-THF/E, THF/F, and THE/E) increased (with the exception of 5 $\alpha$ -reductase activity) between stages A and B and decreased between stages B and C. In boys, these parameters did not change during pubertal development. In both sexes, CYP3A4 activity (6-OH cortisol/cortisol ratio) was stable across pubertal development.

## Discussion

In this longitudinal study, we have demonstrated that the excretion of cortisol metabolites diverges between sexes with advancing pubertal maturation. Therefore, our study suggests that the sexual dimorphism in cortisol metabolism that generally starts around the age of 11

is a hormonally driven process, either directly, by influencing gene expression, or indirectly, by impacting on body composition.

Previous studies showed that adult men and women differ in the excretion rate of cortisol metabolites, which was higher in males (13, 45). Wudy *et al.* found that these differences emerged from the age of 11 to 12 years (18), suggestive of an important role of gonadal hormones. However, their study lacked an assessment of pubertal status. In our study, which included repeated assessment of Tanner pubertal stage, we found that the excretion rate of cortisol metabolites decreased significantly in girls between early and late-pubertal stage, suggestive of an effect of pubertal development on the excretion rate of cortisol metabolites in girls. In contrast to previous research (18), we were not able to detect statistically significant differences in cortisol metabolite excretion rate between sexes which might be due to the use of morning instead of 24-h urine or the relatively small sample size.

Our data provided evidence for a sexual dimorphism in cortisol metabolism, as assessed by ratios reflecting the activities of the enzymes involved. We found that with advancing pubertal maturation in females, as compared to males, the balance of 11 $\beta$ -HSDs progressively favored cortisone and that the proportion of A-ring reduced metabolites was lower, in line with data in adults (13, 14, 15, 20, 45). During pubertal maturation, levels of sex steroids increase gradually along with the development

**Table 5** Changes in cortisol metabolite excretion rate and cortisol metabolite ratios during pubertal development.

Parameter	Pubertal stage <sup>a</sup>	Girls Mean difference (95% CI)	P value	Boys Mean difference (95% CI)	P value
Cortisol metabolite excretion rate	A → B	-0.004 (-0.069–0.060)	0.913	-0.059 (-0.160–0.012)	<b>0.040</b>
	B → C	-0.128 (-0.188–0.068)	<b>&lt;0.001</b>	-0.024 (-0.097–0.048)	0.580
5 $\alpha$ -reductase activity	A → B	0.928 (-0.234–2.090)	0.189	-1.150 (-2.367–-1.150)	0.120
	B → C	-4.406 (-5.703–-3.109)	<b>&lt;0.001</b>	0.303 (-0.945–-0.303)	0.690
5 $\beta$ -reductase activity (a)	A → B	1.953 (1.097–2.808)	<b>&lt;0.001</b>	1.172 (-0.209–2.553)	0.163
	B → C	-1.325 (-2.366–-0.284)	<b>0.036</b>	-0.638 (-2.113–0.837)	0.477
5 $\beta$ -reductase activity (b)	A → B	4.416 (1.501–7.331)	<b>0.013</b>	2.056 (-0.359–4.471)	0.161
	B → C	-5.422 (-8.335–-2.508)	<b>0.002</b>	1.003 (-2.180–4.186)	0.604
11 $\beta$ -HSD type 2 activity	A → B	-0.124 (-0.213–-0.036)	<b>0.021</b>	0.043 (-0.025–0.111)	0.302
	B → C	0.492 (0.363–0.620)	<b>&lt;0.001</b>	0.326 (0.220–0.433)	<b>&lt;0.001</b>
Balance of 11 $\beta$ -HSD activities	A → B	-0.061 (-0.119–-0.004)	0.079	0.016 (-0.031–0.063)	0.566
	B → C	0.117 (0.046–0.188)	<b>0.007</b>	0.153 (0.089–0.218)	<b>&lt;0.001</b>
Cytochrome P450 3A4 activity	A → B	0.013 (-0.205–0.232)	0.919	-0.121 (-0.315–0.073)	0.304
	B → C	-0.134 (-0.400–0.133)	0.410	0.025 (-0.251–0.301)	0.883

<sup>a</sup>A = pre-pubertal (Tanner stage 1); B = early pubertal (Tanner stage 2–3); C = late-pubertal (Tanner stage 4–5).

of secondary sexual characteristics (46). Therefore, our findings suggest that sex steroids influence cortisol metabolism either directly, for example, by influencing gene expression, or indirectly, by impacting on body composition (47, 48, 49).

With advancing pubertal development, differences in body composition emerge between sexes; girls gain more mass and boys acquire more fat-free mass and skeletal mass (50). These differences are regulated by endocrine factors, including gonadal steroids and growth hormone (51), in addition to genetic and environmental factors. Body composition is strongly associated with HPA-axis activity, both in adulthood and childhood (52, 53), and the observed differences between sexes in cortisol parameters that emerge during pubertal development could (partially) be explained by progressive differences in body composition. Although males and females did not differ in BMI at any age, unfortunately our study lacked a more detailed assessment of body composition.

It is unclear whether the sex differences in cortisol metabolism that we observed are androgen- or estrogen mediated. A study in adult men showed that testosterone reduced the CRH-stimulated rise in serum cortisol, in spite of increased ACTH, suggestive of adrenal hyporesponsiveness (54). Evidence for an effect of androgens on glucocorticoid metabolism was provided by other studies (55, 56, 57). It has been demonstrated by multiple studies that women with polycystic ovary syndrome – a condition characterized by increased androgen production – had a higher 5 $\alpha$ -reductase activity than BMI-matched controls (55). However, this may be part of the PCOS trait rather than an effect of hyperandrogenism, as their daughters already had a greater 5 $\alpha$ -reductase activity, in spite of a similar androgen metabolite excretion rate, from early

childhood than age-matched controls (56). In contrast, experiments in gonadectomized male rats suggested that androgens suppress the expression and/or the activity of hepatic A-ring reductases (57). In addition, the same experiments suggested that androgens increase 11 $\beta$ -HSD type 1 in liver and adipose tissue (57).

Studies in rats and humans suggest that estrogens could also influence glucocorticoid metabolism, though findings were contradictory. Several studies in rodents have shown that the expression and/or the activity of A-ring reductases and 11 $\beta$ -HSD type 1 was upregulated and downregulated by estrogen, respectively (19, 22, 58, 59, 60, 61). In contrast, in humans the activity of these enzymes was independent of the phase of the menstrual cycle or, after menopause, use of estrogen replacement therapy (14, 15, 20).

Our study has several strengths and limitations. The major strength of our study was the long follow-up period of 8 years, enabling us to assess the contribution of pubertal maturation to sexual dimorphism in HPA-axis functioning. Moreover, participants were recruited from a population-based twin register and the numbers remaining into follow-up were relatively high for an age group that is notoriously difficult to engage in longitudinal studies. Another strength is that all measurements were performed in the same laboratory at the same time. Samples were frozen as soon as possible and were thawed only once prior to analysis, which enhances stability (62).

Our study also has its limitations. First, the Brain Scale study was not powered specifically for the present study and, hence, a sample size calculation was not performed prior to analysis, which might explain our inability to detect differences in cortisol metabolite excretion rate between sexes. Second, some limitations related to our



outcome should be acknowledged. Enzymatic activities were estimated by ratios of metabolites in morning urine, which estimate activity only globally. Since cortisol is secreted in a circadian rhythm, a 24-h-urine sample would have been preferred. In addition, both short- and long-term stability of cortisol production and metabolism are low, which could have influenced our results (63, 64). Third, a residual age effect could not be excluded. In order to distinguish between a residual age effect and the effect of pubertal maturation, a larger sample size is warranted. Fourth, Tanner pubertal stages were, for an important part, assessed by self-report, which might result in misclassification, notably under classification (31). Nevertheless, our approach (i.e. classifying according to pre-, early, and late-pubertal stages and the use of the Markov model) is likely to reduce misclassification.

## Conclusion

With advancing pubertal maturation, sexual dimorphism in cortisol metabolism became increasingly manifest, while no differences were seen prepubertally. These differences could emerge from direct or indirect effects of sex steroids on cortisol metabolism.

### 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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