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Modified release and conventional glucocorticoids and diurnal androgen excretion in congenital adrenal hyperplasia

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ABSTRACT

Context: The classic androgen synthesis pathway proceeds via DHEA, androstenedione and testosterone to 5α-dihydrotestosterone (DHT). However, DHT synthesis can also be achieved by an alternative pathway originating from 17α-hydroxyprogesterone (17OHP), which accumulates in congenital adrenal hyperplasia (CAH). Similarly, recent work has highlighted androstenedione-derived 11-oxygenated 19-carbon steroids as active androgens and, in CAH, androstenedione is generated directly from 17OHP. The exact contribution of alternative pathway activity to androgen excess in CAH and its response to glucocorticoid therapy is unknown.

Objective: We sought to quantify classic and alternative pathway-mediated androgen synthesis in CAH, their diurnal variation and their response to conventional glucocorticoid (GC) therapy and modified release hydrocortisone.

Methods: We employed urinary steroid metabolome profiling by gas chromatography-mass spectrometry for 24-h steroid excretion analysis, studying the impact of conventional GCs (hydrocortisone, prednisolone, dexamethasone) in 55 adults with CAH and 60 controls. We studied diurnal variation in steroid excreton by comparing 8-hourly collections (23:00-7:00h, 7:00-15:00h, 15:00-23:00h) in 16 CAH patients on conventional glucocorticoids and during six months of treatment with modified release hydrocortisone, Chronocort.

Results: CAH patients on conventional GCs showed low excretion of classic pathway androgen metabolites but excess excretion of the alternative pathway signature metabolites 3α,5α-17-hydroxypregnanolone and 11β-hydroxyandrosterone. Chronocort reduced 17OHP and alternative pathway metabolite excretion to near normal levels more consistently than other GC preparations.

Conclusions: Alternative pathway mediated androgen synthesis significantly contributes to androgen excess in CAH. Chronocort therapy appears superior to conventional GC therapy in controlling androgen synthesis via alternative pathways through attenuation of their major substrate, 17OHP.

INTRODUCTION

Disruption of glucocorticoid (GC) synthesis is the defining feature of all variants of congenital adrenal hyperplasia (CAH) including its most prevalent cause, 21-hydroxylase deficiency (21OHD) (1). This enzymatic block results in GC deficiency, with the consequent loss of negative feedback to the pituitary gland and hypothalamus, driving both ACTH-mediated adrenal androgen excess and adrenal hyperplasia. Mineralocorticoid deficiency may also be seen in 21OHD, but to a variable degree dependent on mutation severity (2).

The classic pathway of androgen synthesis proceeds through dehydroepiandrosterone (DHEA), androstenedione and testosterone to the most potent activator of the androgen receptor, 5α-dihydrotestosterone (DHT). The substrate of 21-hydroxylase, 17α-hydroxyprogesterone (17OHP), accumulates in CAH due to 21OHD, resulting in enhanced conversion to androstenedione and active androgens. However, 17OHP is also a substrate for an alternative pathway to androgen biosynthesis, which generates DHT without the need for DHEA, androstenedione or testosterone as intermediates (3, 4). In this pathway, 17OHP is converted by consecutive 5α-reductase and 3α-HSD activity to 3α,5α-17-hydroxypregnanolone (3α,5α-17HP) and then downstream to DHT (**Fig. 1**) (5). Accumulation of the alternative pathway intermediate 3α,5α-17HP has been demonstrated in untreated patients with CAH due to 21OHD (6), but its relative contribution to excess androgen synthesis has not yet been investigated. Furthermore, recent work has highlighted the role of another androgen biosynthesis pathway that converts androstenedione in several steps to 11-keto-testosterone and 11-keto-dihydrotestosterone (**Fig. 1**), steroids that have been shown to act as potent androgen receptor agonists (7-11).

Conventional management strategies for CAH include the use of both immediate release hydrocortisone and longer-acting synthetic GC preparations, sometimes prescribed in a reverse circadian pattern (1). These preparations fail to mimic the normal diurnal profile of cortisol secretion and therefore do not prevent the early morning surge of ACTH that is the major driver of adrenal-mediated androgen excess in CAH. As a consequence, the current management of patients with CAH is complicated by the need to strike a balance between sufficient control of endogenous androgen excess and potential excess

exposure to exogenous GCs (12). A modified and delayed release GC preparation, Chronocort, has recently been developed and shown to approximate the physiological diurnal rhythm of cortisol release due to delayed release, with peak levels during the early morning hours after intake at bedtime (13, 14). The relative impact of both conventional GC preparations and Chronocort on androgen synthesis by classic and alternative pathways is not known.

In this study, we sought to quantify the diurnal contribution of alternative pathway androgen synthesis to androgen excess in CAH by assessing the excreted urinary steroid metabolome of 21OHD patients. We investigated patients receiving conventional GC therapy and patients treated with the modified release hydrocortisone preparation Chronocort in comparison to healthy controls with intact diurnal secretion of cortisol.

SUBJECTS AND METHODS

Subjects

Alternative pathway androgen synthesis in subjects with CAH managed with conventional GC therapy was quantified by analysis of 24-hour urinary steroid metabolite excretion in 55 adult subjects with 21OHD, recruited from two specialist centers, Birmingham and Munich, and 60 sex- and age-matched controls, recruited from Birmingham. In all participating patients, the diagnosis of 21-hydroxylase deficiency had previously been confirmed following genetic testing as part of their routine clinical care. Control subjects were healthy individuals without chronic disease aged 18-80 years. None were taking oral contraceptives, hormone replacement therapy other than corticosteroid replacement or other medications known to alter steroid hormone synthesis and/or metabolism at the time of urine collection.

A summary of patient and control characteristics is provided in **Table 1**. The majority of the 21OHD group were managed with prednisolone (n=27; 49%; median daily dose 7.5 mg, range 5-15 mg) and the remainder with either hydrocortisone (n=13; 24%; median daily dose 30 mg, range 20-37.5 mg) or dexamethasone (n=15; 27%; median daily dose 0.5 mg, range 0.25-1.00 mg). All patients with salt-wasting CAH and some

with simple-virilizing CAH received additional mineralocorticoid replacement; daily fludrocortisone doses ranged between 100-300 μg.

The impact of the modified release hydrocortisone preparation Chronocort on alternative pathway synthesis was assessed in a subgroup consisting of 16 subjects with 21OHD. All were enrolled in an open-label phase 2 study at the National Institutes of Health Clinical Centre (clinical trials.gov #NCT01735617) (14). Subjects were maintained on twice-daily Chronocort therapy for a period of six months with dose adjustment employed based on clinical symptoms and serum biochemistry. Median daily Chronocort dose at six months was 27.5 (range 15-40) mg. Urinary steroid metabolite excretion was measured at baseline, at day four of Chronocort therapy and after six months of treatment. Three eight-hour urine samples were collected within each of these three 24 hour periods and were timed to reflect either night (23:00-07:00), morning (07:00-15:00) or evening (15:00-23:00) periods. Steroid excretion in the 8-hourly urine collection was compared to that of 12 healthy control subjects (median age 32.9 years) who also provided three eight-hour urine collections with similar timing to reflect night, morning or evening periods. All participants provided informed written consent. Ethical approval for the collection of baseline data was provided by South Birmingham Research Ethics Committee (REC) for healthy controls, and by West Midlands MREC and the University Hospital Ethics Committee Munich for conventionally managed CAH patients. Phase 2 study approval for the Chronocort-treated CAH patients was provided by the Eunice Kennedy Shriver National Institute of Child Health and Human Development Institutional Review Board at the National Institute of Health, USA.

Urinary steroid hormone analysis

Analysis of urinary excretion of steroid hormone metabolites was undertaken by quantitative gas chromatography-mass spectrometry (GC-MS) in selected-ion-monitoring analysis mode as described previously (7). **Suppl. Table 1** summarizes the steroid metabolites relevant to this study.

The 21-hydroxylase enzyme, CYP21A2, catalyzes the conversion of 17OHP to the cortisol precursor 11-deoxycortisol. The metabolic impact of 21OHD was thus assessed through analysis of tetrahydro-11-deoxycortisol (THS), the metabolite of the CYP21A2 product 11-deoxycortisol, and the

17OHP metabolites 17-hydroxypregnanolone (17HP) and pregnanetriol (PT) as well as pregnanetriolone (PTONE). PTONE is the metabolite of 21-deoxycortisol, which is generated from 17OHP by CYP11B1 and only produced in appreciable amounts in the absence of 21-hydroxylase activity, i.e. in 21OHD.

Classic androgen pathway activity was measured by quantification of the major androgen metabolites androsterone (An) and etiocholanolone (Et). Activation of the alternative pathway to DHT was assessed through quantification of its signature metabolite 3α,5α-17HP. While androstenedione and testosterone both feed into An and Et, the most potent androgen, DHT, is only represented in the 5α-reduced androgen metabolite An. Thus the Et pool is only enhanced by classic androgen pathway activity while the An pool increases with DHT synthesis via both the classic and alternative pathways. We therefore used the ratio 3α5α17HP/An as an estimate of the proportional contribution of the alternative pathway to androgen synthesis.

19-carbon androgens oxygenated at position C-11 have been shown to be produced by the adrenal glands and 11-keto-testosterone and 11-keto-DHT have been shown to activate the androgen receptor (7, 10). Therefore, we measured the concentration of the major metabolite of urinary 11-oxy-C_{19} steroid metabolites, 11β-hydroxyandrosterone (11β-OH-An).

Statistical analysis

Data are presented as median and interquartile range (IQR) unless otherwise stated. Analyses were undertaken using the non-parametric Mann-Whitney and Kruskal-Wallis with posthoc Dunn tests for unpaired analyses. Paired data was analyzed using the non-parametric Wilcoxon test with Bonferroni correction applied for repeated analyses. Statistical analyses were undertaken using SPSS Statistics 21 (IBM) and p-values <0.05 considered significant. All p-values were two sided.

RESULTS

24-h steroid metabolite excretion in CAH patients receiving conventional glucocorticoid therapy

As expected, the excretion of the 17OHP metabolites 17HP and PT and the 21-deoxycortisol metabolite PTONE, were significantly increased in CAH ($p<0.001$), indicative of impaired 21-hydroxylase activity. Conversely, the product of 21-hydroxylase activity, the 11-deoxycortisol metabolite THS, was significantly lower ($p<0.001$) in subjects with CAH than in control subjects (**Fig. 2A+B**).

The urinary excretion of the sum of the major androgen metabolites An and Et was significantly lower in subjects with CAH managed with conventional GC therapy than in sex- and age-matched control subjects (**Fig. 2C**; $p<0.001$). Conversely, the signature metabolite of the alternative pathway to DHT synthesis, 3α5α-17HP, was significantly increased in subjects with CAH (**Fig. 2D**; $p<0.001$). The ratio of 3α,5α-17HP to An was calculated in order to quantify the contribution of the alternative pathway to total synthesis of 5α-reduced androgens including DHT. This ratio was significantly increased in subjects with CAH while alternative pathway activity in the controls was negligible ($p<0.001$) (**Fig. 2E**). The excretion of the major metabolite of 11-oxygenated 19-carbon steroids, 11β-OH-An, appeared similar to that in controls, with broad inter-individual variability in excretion amounts (**Fig. 2F**). The pattern of changes remained similar when carrying out sex-specific sub group analyses (**Suppl. Fig. 3**), which also revealed higher excretion of the metabolites of 17OHP (**Suppl. Fig. 3A**) and classic and alternative pathway metabolites (**Suppl. Fig. 3C,D,F**) in male controls as compared to female controls, whereas no significant difference was observed between male and female CAH patients.

Diurnal variation in steroid excretion in CAH patients receiving conventional glucocorticoid therapy

Diurnal excretion analysis in urines collected in 8-hour intervals reflecting night (23:00-07:00h), morning (7:00-15:00h) and evening (15:00-23:00h) showed a similar picture to the 24-h urine analysis when comparing CAH patients (n=16; four of whom were managed with hydrocortisone, seven with prednisolone and five dexamethasone) to control subjects (n=12), with lower classic pathway but higher excretion of the signature metabolite of the alternative pathway to DHT in CAH patients (**Fig. 3**).

Healthy control subjects showed significant diurnal variability of the metabolites of 17OHP and the classic and alternative androgen pathways (**Fig. 3**), with lowest excretion during night time. By contrast, this diurnal excretion pattern was lost in CAH patients receiving conventional glucocorticoid therapy.

Differential impact of conventional glucocorticoid preparations on steroid excretion

To assess the effect of distinct conventional GC therapies on androgen synthesis, we compared urinary steroid metabolite excretion in CAH patients managed with hydrocortisone (n=13), prednisolone (n=27), and dexamethasone (n=15), respectively; all had been on stable treatment for at least six months. This revealed that hydrocortisone-treated CAH patients had significantly higher excretion of 17OHP metabolites, the sum of the androgen metabolites An+Et and also the major adrenal androgen metabolite 11β-OH-An in comparison to dexamethasone-treated patients, with prednisolone-treated in an intermediate position (**Suppl. Fig. 1**). Similarly, hydrocortisone therapy appeared associated with the highest excretion of the alternative pathway metabolite 3α,5α-17HP but this difference was not statistically significant due to high inter-individual variability.

Diurnal steroid excretion during modified release hydrocortisone treatment

We assessed urinary steroid excretion in 16 patients with CAH at baseline, i.e. on conventional GC treatment, and during treatment with modified release hydrocortisone, with diurnal urine collections in 8-hourly intervals. This was carried out on three occasions: at baseline when still receiving conventional GC therapy, shortly after initiation of Chronocort treatment, day 4, and after six months of continuous treatment with Chronocort.

The analysis of the total 24-h urine excretion revealed a significant reduction in the combined excretion of the markers of impaired 21-hydroxylase activity, the sum of 17α-hydroxyprogesterone metabolites 17HP, PT and the 21-deoxycortisol metabolite PTONE, both after four days and six months of Chronocort treatment (all $p<0.05$) (**Fig. 4**), with lower excretion amounts than observed in patients treated with any other GC preparation (**Fig. 5**). Total classic pathway androgen metabolite excretion, An+Et, and excretion of the alternative androgen pathway metabolite 3α5α-17HP significantly decreased after

Chronocort treatment to lower levels than observed with any other GC preparation (**Fig. 4+5**). The excretion of 11β-OH-An also appeared to decrease albeit not significantly (**Fig. 4**). Attenuation of 11-oxygenated 19-carbon androgen synthesis in Chronocort-treated patients was at least similar to dexamethasone or prednisolone treatment and superior to the effects of hydrocortisone treatment (**Fig. 5**). Of note, 24-h urinary excretion of 11-hydroxy-etiocholanolone and 11-oxo-etiocholanolone, which are exclusive glucocorticoid metabolites (15) and therefore reflective of the amount of exogenous cortisol, showed a higher excretion in patients treated with conventional hydrocortisone than in patients on Chronocort.

The effect of six months of Chronocort therapy on the diurnal rhythm of urinary steroid excretion in subjects with CAH is shown in **Suppl. Fig. 2**. There was less variability seen across the three 8-hour periods in the excretion of the metabolites of CYP21A2 following Chronocort than prior to its initiation. Notably, the early morning surge (night time period, 23:00-7:00h) in the activation of classic and alternative androgen pathway synthesis appeared diminished following Chronocort therapy (**Suppl. Fig. 2C-F**).

DISCUSSION

In this study, employing 24-h urinary steroid metabolome profiling, we could show that alternative pathway androgen synthesis contributes significantly to androgen excess in CAH patients receiving chronic GC therapy, both via the 11-oxygenated 19-carbon androgen pathway and via DHT synthesis from 17OHP. In addition, we have identified the differential impact of conventional GC therapies and treatment with modified release hydrocortisone (Chronocort) on steroid excretion in CAH, including their effects on alternative pathway androgen synthesis, namely the alternative "backdoor" pathway to DHT and the 11-oxygenated C19 steroid pathway.

Eleements of the alternative "backdoor" pathway to DHT were first described by Wilson, Auchus and colleagues, reporting the synthesis of 5α-androstanediol from 17OHP, with 3α,5α-17HP as the intermediate in the fetal testis of the tammar wallaby pouch young (5). They hypothesized that this pathway could extend to the conversion of 5α-androstanediol to 5α-dihdyrotestosterone (DHT), thereby achieving active androgen synthesis without the classic pathway intermediates DHEA, androstenedione and

testosterone. This led Auchus to coin the term "backdoor pathway" for this alternative pathway to DHT synthesis (3). They later showed that the final step to DHT can indeed take place in the gonads of the brushtail possum, the tammar wallaby, and the short tail opossum (16-18). Arlt and colleagues were the first to suggest the relevance of the alternative pathway to DHT in humans, as an explanation for the virilization of newborn girls affected by CAH, utilizing the example of CAH due to P450 oxidoreductase deficiency, which results in disruption of the classic androgen pathway (4). Though that work focused on the role of the alternative pathway in prenatal life, they postulated that synthesis of DHT via the alternative pathway is likely to occur or increase, respectively, if there is an increase in either the availability of its substrate 17OHP or the activity of 5α-reductase type 1 activity, which catalyzes the first step of the alternative pathway. Both progesterone and 17OHP are efficient substrates for the 5α-reductase activity of SRD5A1 (19) and both these steroids accumulate in CAH with impaired 21-hydroxylase activity. Ogata's group showed increased urinary excretion of the alternative pathway intermediate 3α,5α-17HP in patients with CAH due to P450 oxidoreductase deficiency (20); P450 oxidoreductase serves as the electron donor enzyme to 21-hydroxylase and therefore its disruption results in impaired 21-hydroxylase activity. Subsequently, Kamrath et al. demonstrated increased 3α,5α-17HP in newly diagnosed and hence untreated patients with CAH due to 21OHD aged 1 day to 25 years, noting the highest excretion amounts in the neonatal period (6). In this study investigating the steroid metabolome in adult CAH patients on established GC therapy, we found that while classic androgen pathway activity was significantly reduced, there was significantly increased excretion of 3α,5α-17HP, indicating an increased relative contribution of alternative androgen pathway DHT synthesis to androgen excess in CAH also in adulthood and in patients receiving regular GC treatment.

We also found increased excretion of 11β-OH-An, the major metabolite of 11-oxygenated 19-carbon androgens. Of note, Kamrath et al. also showed significantly increased excretion of 11β-OH-An in untreated CAH patients. However, at the time, they considered 11β-OH-An a classic pathway metabolite, while in fact this steroid represents the major metabolite of 11β-hydroxy-androstenedione and other 11-

oxygenated 19-carbon androgens (15), effectively the second alternative pathway to the synthesis of active androgens. Its end products, 11-keto-testosterone and 11-keto-DHT, which have shown similar androgenic activity to testosterone and DHT (7-10). In a very recent publication, serum metabolome profiling by tandem mass spectrometry demonstrated 3-4 fold increased circulating concentrations of 11β-hydroxy-androstenedione, 11-keto-androstenedione, 11β-hydroxy-testosterone, and 11-keto-testosterone in patients with 21OHD (21). However, this was done in a cross-sectional cohort of CAH patients with no detailed data on GC therapy available.

In our study, conventional GC therapy appeared to control the activity of the alternative androgen synthesis pathways less efficiently than classic pathway synthesis. The latter we even found to be significantly suppressed in CAH patients, below the levels observed in healthy sex- and age-matched controls, indicative of relative GC over-treatment that is frequently observed in adult patients with CAH (22). Studying the diurnal variation of steroid excretion in our patients, we observed that the increased excretion of the alternative pathway metabolites 3α,5α-17HP and 11β-OH-An is most likely consequent to the early morning surge in ACTH, which is unopposed in CAH patients on conventional GC therapy.

By contrast, we found that Chronocort, a modified release hydrocortisone preparation, exerted much improved control of alternative pathway-mediated androgen excess. Chronocort has been shown to yield cortisol delivery mimicking physiological cortisol secretion (13), resulting in significant normalization of circulating 17OHP and androstenedione levels in a previously published phase 2 study in CAH patients (14). This effect was almost more impressively visible when studying the urines of this cohort of 16 patients in our study, with close to normalization of 17OHP metabolite excretion in Chronocort-treated patients. Conventional GC treatment never normalizes 17OHP secretion and if present, this would be considered an indicator of significant over-replacement. However, near normal diurnal provision of cortisol by Chronocort exerted superior control of 17OHP secretion and thereby also of both alternative androgen pathways driving androgen excess in CAH, which are both fed by the conversion of 17OHP, either to 11-oxygenated 19-carbons steroids or to 3α,5α-17HP and further downstream to DHT via the

"backdoor pathway". An alternative modified release formulation of hydrocortisone, Plenadren, has immediate and delayed release actions but is licensed for use in adrenal insufficiency, where it is taken first thing the morning as a once daily medication, (23) and was not studied here. In the only paper to report use of Plenadren in CAH, six patients with CAH were included in an open label trial of Plenadren where BMI, HbA1c and quality of life were measured but androgens were not reported.(24)

Importantly, in our study, the analysis of the exclusive cortisol metabolites 11β-hydroxy-etiocholanolone and 11-oxo-etiocholanolone (15) clearly indicated a higher excretion in the patients on conventional hydrocortisone treatment than in those treated with Chronocort, the modified release hydrocortisone preparation. This means that the absolute amount of bioavailable cortisol was actually lower in Chronocort-treated CAH patients, supporting the assumption that it was not the total amount of glucocorticoid but the improved diurnal delivery of cortisol by Chronocort, and therefore the better control of the early morning ACTH and steroid surge, that results in the superior control of excess 17OHP and androgen production.

A limitation of our study was the fact the CAH patient groups receiving the three conventional GC preparations, hydrocortisone, prednisolone and dexamethasone, were not matched for biochemical control at baseline and were studied cross-sectionally and not during a controlled cross-over study. However, they were a cohort of considerable size recruited from two large specialist centers, which ensures a relative homogenization of clinical presentation. An advantage of our study was the inclusion of adult patients only, which allowed us to dissect androgen production in detail.

In conclusion, we have identified significant alternative androgen synthesis pathway activity in adult patients with CAH on conventional GC therapy that persists despite suppression of classic pathway androgen production by relative glucocorticoid overtreatment. However, we found that the modified release hydrocortisone preparation Chronocort results in superior control of alternative pathway androgen production, most likely by reducing the early morning surge in excess 17OHP, which in CAH represents the major substrate for both the alternative androgen pathway to DHT and the 11-oxygenated androgen pathway.

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FIGURE LEGENDS

Fig. 1: Schematic overview of steroidogenesis. The graph depicts steroidogenesis including the classic androgen synthesis pathway (shaded in dark grey) and the two alternative androgen synthesis pathways (shaded in light grey; top, 11-oxygenated 19-carbon steroids; bottom, alternative pathway to DHT). 3α,5α-17HP is labelled by its alternative full name, 17-OH-allopregnanolone.

Fig. 2: 24-h urinary steroid excretion in 55 CAH patients and 60 healthy sex- and age-matched controls. For explanation of steroid metabolite abbreviations see **Table 1**. Data are shown as µg/24 hours and presented as box-and-whisker plots to represent median, interquartile range (box), and 5^{th} and 95^{th} percentiles (whiskers). Urinary excretion of 3α5α-17HP available for 38 of the total CAH cohort. Analyses were undertaken using the Mann-Whitney test. * $p \leq 0.05$, *** $p \leq 0.001$ for CAH vs. controls.

Fig. 3: 8-hourly diurnal urinary steroid metabolite excretion in 16 subjects with CAH due to 21OHD and 12 healthy controls. Data are shown for night (23:00-07:00; dark grey), morning (07:00-15:00; white) and evening (15:00-23:00; light grey) time periods. Excretion of the major androgen metabolites An+Et is shown for male subjects with CAH (n=8) and matched healthy controls (n=12). Box-and-whisker plots represent median, interquartile range (box), and 5^{th} and 95^{th} percentiles (whiskers). Comparisons were drawn within CAH and control groups with analyses undertaken using the Friedman test, which was applied to the CAH subjects and the control subjects separately. * $p \leq 0.05$ for comparison of steroid excretion during different 8-h periods.

Fig. 4: Effect of Chronocort treatment on 24-hour urinary steroid metabolite excretion in subjects with CAH due to 21OHD. Results are shown for subjects with CAH at baseline on conventional GC therapy prior to commencing Chronocort (BL; n=16), at day 4 of Chronocort treatment (D4; n=16) and after six months of Chronocort treatment (M6; n=15). Box-and-whisker plots represent median,

422 interquartile range (box), and 5th and 95th percentiles (whiskers). Analyses were undertaken using repeated
423 Wilcoxon tests with Bonferroni correction to compare between matched CAH subjects. * $p \leq 0.05$.
424
425 **Fig. 5: Urinary steroid excretion in 60 healthy controls and CAH patients treated with Chronocort**
426 **(n=16), conventional immediate release hydrocortisone (n=13), prednisolone (n=27) or**
427 **dexamethasone (n=15).** Urinary excretion of 3α5α-17HP available for 54 of the total CAH cohort; 16 on
428 Chronocort, 11 on conventional hydrocortisone, 21 on prednisolone and 6 on dexamethasone.
429 Glucocorticoid treatment was stable for at least six months at the time of 24-h urine collection. Box-and-
430 whisker plots represent median, interquartile range (box), and 5th and 95th percentiles (whiskers). Analyses
431 were undertaken using the Kruskal-Wallis test with post-hoc Dunn. * $p \leq 0.05$, *** $p \leq 0.001$.
432

433 **Table 1:** Demographic data for participants with CAH managed with either conventional glucocorticoid
434 (GC) treatment or Chronocort and healthy matched control subjects. Data for age are shown as median
435 (range). For Chronocort-treated patients their conventional GC medication prior to commencing
436 Chronocort therapy is shown.

		CAH patients on conventional GC therapy	**Control cohort**	**Chronocort-treated CAH patients**	**Control cohort**
Number		**N=55**	**N=60**	**N=16**	**n=12**
Sex	**Male : Female** n:n	28 : 27	32 : 28	8 : 8	12 : 0
Age (years)		31 (19-49)	26 (20-48)	24 (18-60)	28.5 (22-60)
CAH phenotype	**Salt-wasting** (%)	41 (74.5)	-	12 (75)	-
	Simple virilizing (%)	14 (25.5)	-	4 (25)	-
Glucocorticoid preparation	**Hydrocortisone** n (%)	13 (24)	-	3 (19)	-
	Prednisolone n (%)	27 (49)	-	8 (50)*	-
	Dexamethasone n (%)	15 (27)	-	5 (31)	-

437

438 * One patient received a combined hydrocortisone and prednisolone preparation prior to commencing
439 Chronocort therapy and has been included in the prednisolone group

Fig. 1: Schematic overview of steroidogenesis.

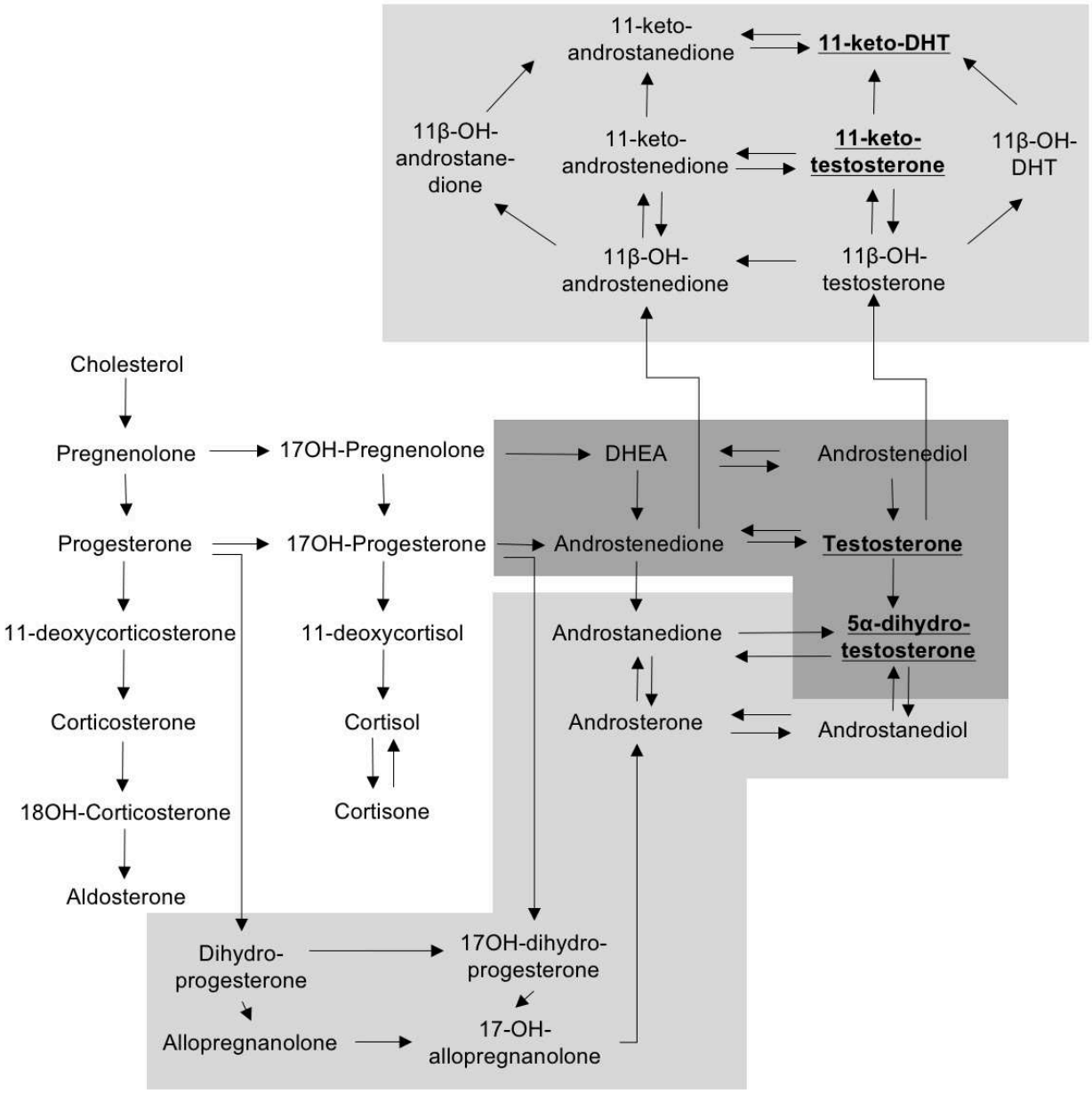

Fig. 2: 24-h urinary steroid excretion in 55 CAH patients and 60 healthy sex- and age-matched controls.

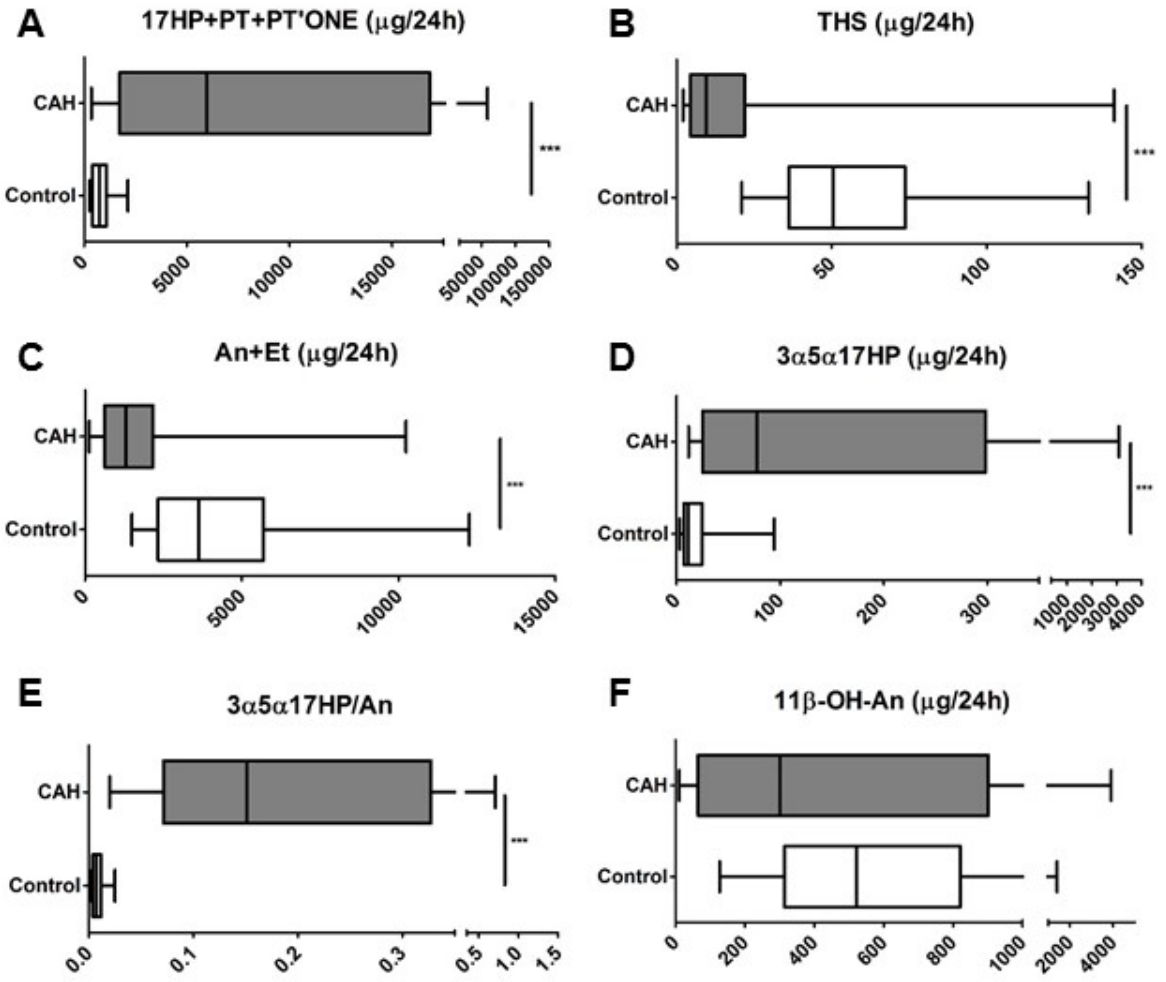

Fig. 3: 8-hourly diurnal urinary steroid metabolite excretion in 16 subjects with CAH due to 21OHD and 12 healthy controls.

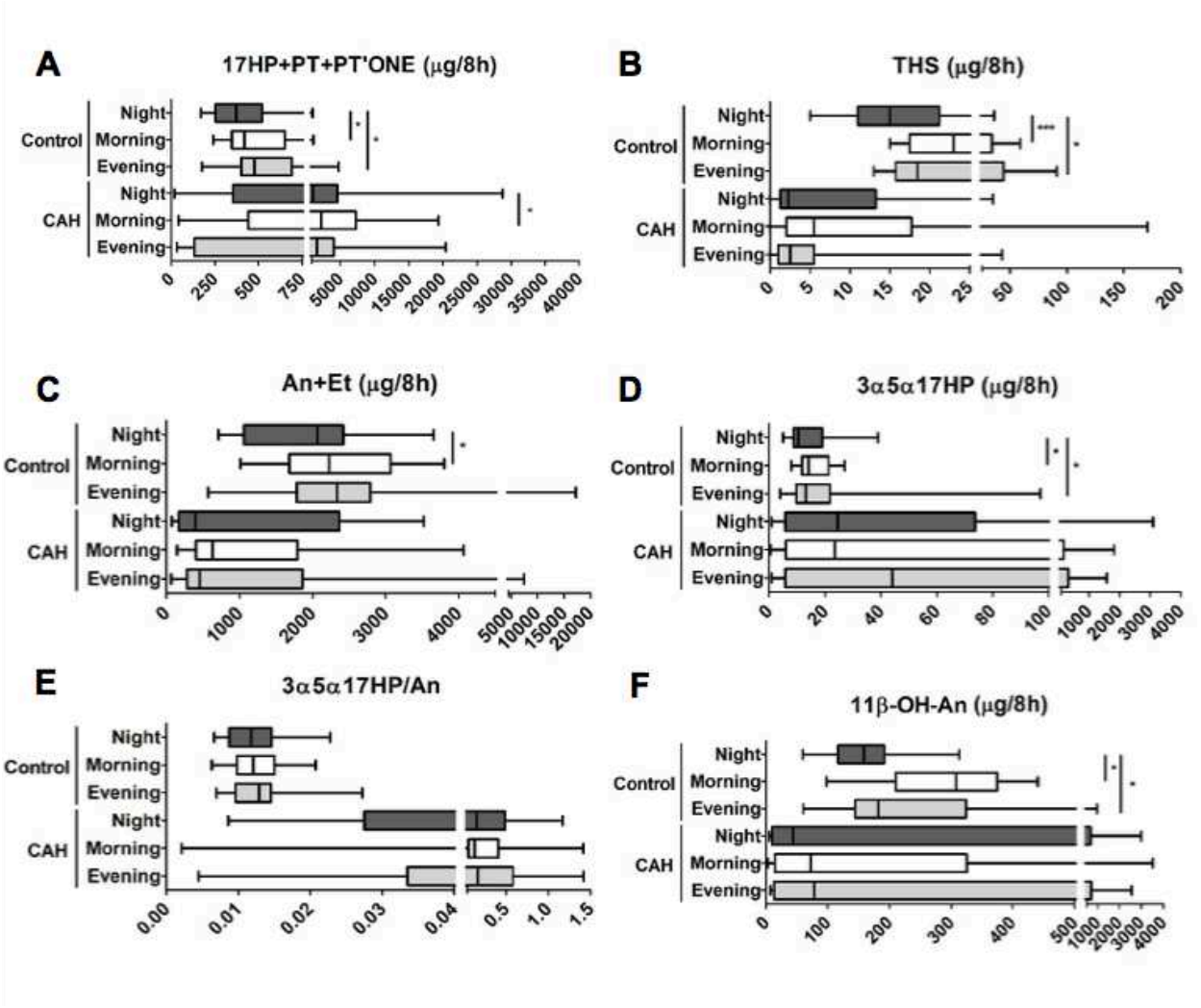

Fig. 4: Effect of Chronocort treatment on 24-hour urinary steroid metabolite excretion in 16subjects with CAH due to 21OHD.

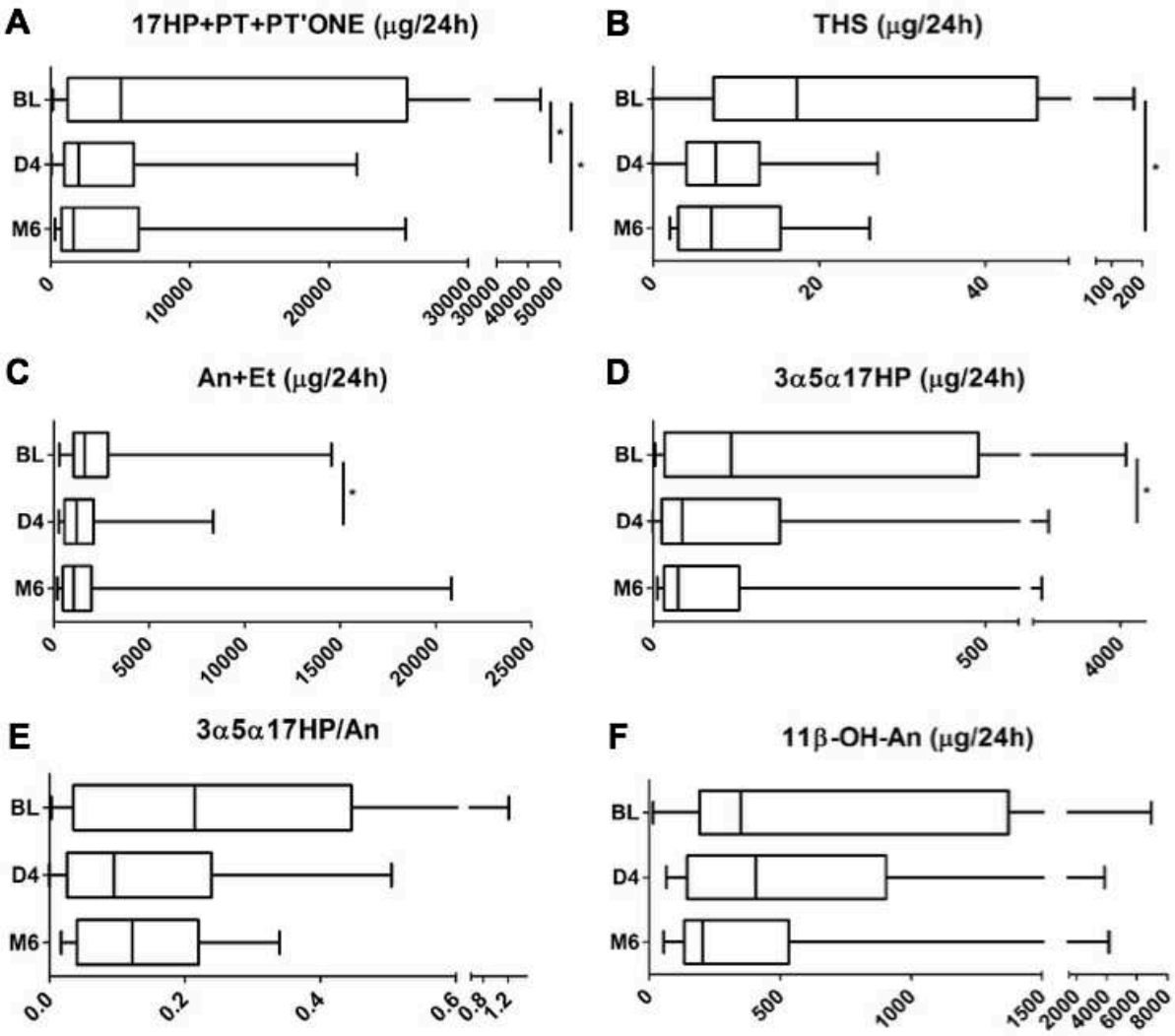

Fig. 5: Urinary steroid excretion in 60 healthy controls and CAH patients treated with Chronocort (n=16), conventional immediate release hydrocortisone (n=13), prednisolone (n=27) or dexamethasone (n=15).