Modelling adrenal steroid profiles to inform monitoring guidance in congenital adrenal hyperplasia

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Summary

Background There is no consensus on how to monitor adrenal androgens in Congenital Adrenal Hyperplasia (CAH).

Methods Modelling of serum and salivary steroid profiles in healthy participants and patients with CAH randomised to either standard treatment or modified-release hydrocortisone hard capsules (MRHC).

Findings Changes in serum 17-hydroxyprogesterone (17OHP) and androstenedione (A4) paralleled each other in healthy participants (n = 19) and patients with CAH (n = 122). However, healthy participants had similar absolute levels of 17OHP and A4 whereas patients with CAH had proportionally higher levels of 17OHP. Cross-correlation showed no lag between serum 17OHP and A4. In CAH, Bayesian multiple change point analysis converged on a 17OHP of 4.5 nmol/l below which in proportion to 17OHP the A4 is lower. Patients on standard treatment had a morning peak in 17OHP and A4 whereas patients on MRHC had relatively flat profiles. Salivary androgens including 11-ketotestosterone correlated with serum 17OHP and A4 in female patients (r = 0.7 to 0.9).

Interpretation In CAH, elevated 17OHP drives the production of A4. High A4 reflects poor control, but low A4 does not indicate overtreatment. Accepting 17OHP is higher than A4, both measurements give similar reflection of control, and a 17OHP <38 nmol/l (1250 ng/dl) was associated with an A4 in the normal range <5 nmol/l (143 ng/dl) in 95% of patients and in clinical trials was used to define good control. On MRHC, which controls androgen levels over 24 h, a single sample of 17OHP and/or A4 can be used to monitor control. Salivary measurements reflect similar results to serum.

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Research in context

Evidence before this study

We searched PubMed from inception to Mar 10, 2024, using the terms "congenital adrenal hyperplasia" AND "17-OH Progesterone" OR "17OHP" OR "androstenedione" OR "11-ketotestosterone" OR "11-KT" OR "monitoring". No language restrictions were applied. From the papers and reviews identified, there is no consensus on what hormones to measure and at what time of day to measure them when monitoring the biochemical control of CAH. The international guidelines simply state: "monitoring treatment through consistently timed hormone measurements relative to medication schedule and time of day." There is therefore a need to define a rational monitoring strategy for patients with CAH.

Added value of this study

We have analysed and modelled a large dataset of steroid profiles with the aim of rationalising monitoring regimens in CAH. The 122-person multicentre phase 3 study of MRHC versus standard treatment with repeated visits (4 × 24-h profiles per participant) has provided 477 profiles. The pattern of steroid profiles within patients is strikingly similar, with cross correlation between them evidencing no time lag between the two most commonly used biomarkers of disease control; 17-hydroxyprogesterone (17OHP) and androstenedione (A4). The change point analysis we have conducted alongside the visualisation of profiles shows that androstenedione is more likely to be low, whilst 17OHP can still show elevated levels. The other androgens, including testosterone in women and 11-oxygenated androgens in both sexes, correlated with changes in 17OHP and A4. The improved androgen control over 24 h on MRHC shows that a single measurement during the day reflects disease control.

Implications of all the available evidence

Our modelling analysis allows us to define monitoring regimens for patients with CAH. In CAH, A4 levels are proportionally lower for the same 17OHP level than those observed in healthy individuals. This means an A4 above the reference range demonstrates poor control, but a normal A4 does not mean tight control, and a low A4 does not imply over-treatment. A 17OHP <38 nmol/l (1250 ng/dl) was associated with an A4 in the normal range <5 nmol/l (143 ng/dl) in 95% of patients and in clinical trials has been used to reflect good control. On MRHC, which controls androgen levels over 24 h, a single daytime sample of 17OHP and/or A4 is sufficient to monitor biochemical control.

Introduction

Congenital adrenal hyperplasia (CAH) is the commonest genetic endocrine disorder,[1] and mutations in the *CYP21A2* gene encoding the enzyme 21-hydroxylase (21-OHD) account for approximately 95% of cases.[2,3] Deficiency in 21-hydroxylase blocks cortisol synthesis resulting in reduced cortisol feedback and consequently increased pituitary adrenocorticotropic hormone (ACTH) release, which in turn promotes the overproduction of adrenal androgens. Patients with CAH therefore have two major problems: cortisol deficiency and androgen excess. In addition, many patients also have mineralocorticoid deficiency as 21-hydroxylase mediates a key step in aldosterone synthesis.

Androgens are generated through three pathways[4]: the classic androgen pathway via dehydroepiandrosterone (DHEA) to testosterone (T) and dihydrotestosterone (DHT); the 11-oxygenated androgen pathway initiated by the conversion of androstenedione (A4) to 11-hydroxyandrostenedione (11OHA4) and yielding the active 11-ketotestosterone (11 KT); and the alternative pathway to DHT, that in health is active in the testis during male development in the fetus and the early neonatal period,[5] but not active in childhood and adults. In CAH, androgen biosynthesis is upregulated due to the accumulation of 17-hydroxyprogesterone (17OHP) prior to the 21-hydroxylase enzyme block. Excess 17OHP results in atypical, enhanced conversion of 17OHP to A4 by CYP17A1 17,20-lyase activity, which physiologically has a much higher preference for the conversion of 17OH-pregnenolone to DHEA. Accumulating 17OHP also drives increased androgen production via the alternative DHT pathway, and increased A4 feeds enhanced 11-oxygenated androgen pathway activity. In patients with CAH on treatment, DHEA levels are usually low,[6] as A4 is generated atypically from 17OHP rather than from DHEA; however, the precise regulatory mechanisms underlying this relative DHEA deficiency are not known.

The goals of treatment in CAH are to replace cortisol deficiency and control androgen excess. The major challenge to achieving these goals is the excessive rise in ACTH overnight as the lack of cortisol feedback is unopposed in patients receiving conventional, immediate-release glucocorticoids. As a consequence, patients often undulate between glucocorticoid overtreatment and androgen excess.[7,8] This poses a problem in deciding what to measure when monitoring glucocorticoid treatment in patients with CAH. The latest guidelines recommend monitoring treatment through consistently

timed hormone measurements relative to medication schedule and time of day.[9] Traditionally 17OHP and androstenedione have been used as markers of adrenal androgen control, but it is now recognised that the 11-oxygenated adrenal androgen pathway contributes to the adrenal androgenic activity[10]; however, their measurement is not available in most clinical centres. Fundamentally, there is no consensus on what hormones to measure, when, how often, and how to interpret their results to optimize the biochemical control of patients with CAH.

Modified-release hydrocortisone hard capsules (MRHC), development name Chronocort (Efmody®, Diurnal, UK) is now available in Europe. Taken at bedtime, hydrocortisone is released during the early morning hours, resulting in an overnight and early morning rise in cortisol that resembles physiological profiles.[11] As part of the phase 3 clinical trial,[11] detailed 24-h serum steroid profiles of 17OHP and A4 were collected on standard treatment and MRHC and in a sub-cohort of patients diurnal salivary samples were collected for steroid analysis by tandem mass spectrometry. This provides a unique dataset facilitating a detailed analysis of the steroid profiles in patients with CAH on standard treatment and MRHC, respectively. We compared these data to 24-h steroid profiles measured in healthy participants to develop rational biochemical monitoring protocols in CAH.

Methods

Patients

Patients had classic 21-OHD-CAH diagnosed in childhood, adequate mineralocorticoid replacement with renin less than 2 times the upper limit of normal (ULN), and were on stable glucocorticoid therapy over the preceding six months. Exclusion criteria included the use of medication interfering with glucocorticoid metabolism, bilateral adrenalectomy and night shift work. We screened 138 patients, 122 were randomised, and 117 completed the study, with 477 24-h steroid profiles available for analysis (Table 1, Appendix pp1–2). Patients underwent blood sampling for 24-h profiles at baseline, 4, 12, and 24 weeks; blood samples for measurement of 17OHP and A4 were taken every 2 h from 15:00 h to 15:00 h the next day, with serum testosterone measured at 07:00 h. A sub-cohort of 9 patients (7 females) from a single centre (Birmingham, United Kingdom) underwent 2-hourly saliva sampling by passive drooling between 07:00 h and 23:00 h. Patients, stratified by baseline glucocorticoid treatment, were randomized to receive MRHC or continue on standard glucocorticoid therapy. MRHC was prescribed as 5, 10 or 20 mg capsules and the initial dose was the hydrocortisone dose equivalent to their baseline therapy (5× for prednis(ol)one and 80× for dexamethasone) with approximately $^{1}/_{3}$ of the daily dose taken at 07:00 h and $^{2}/_{3}$ of the daily dose taken at 23:00 h. Standard treatment was a mixture of regimens that included hydrocortisone, prednisolone, prednisone and dexamethasone. At baseline, 84% of patients were taking a dose of standard glucocorticoid after 18:00 h, and 84% of patients were diagnosed as salt-wasting. At 4 and 12 weeks, dose titrations were made for both treatment groups, using identical rules, following centralized advice by two independent physicians blinded to all data except 24-h steroid profiles and an investigator-completed adrenal insufficiency checklist. The intention of dose adjustment was to optimise control of CAH according to current standard of care which in previous studies was considered a 17OHP <36 nmol/l (1200 ng/dl), equivalent to ~4-fold the ULN depending on the assay (for this study 10.4 nmol/l (300 ng/dl)) and A4 <ULN (7.0 nmol/l (200 ng/dl)[female], 5.2 nmol/l (150 ng/dl)[male]). The blinded titrators considered morning and/or evening dose adjustments of either MRHC or standard glucocorticoid using 17OHP/A4 measurements from the 24-h profile and adrenal insufficiency symptom questionnaire.

Healthy participants

The healthy control group comprised data from 20 healthy participants from a cohort previously reported[12] (Table 1). These 20 participants underwent 24-h frequent serum sampling, with blood drawn in 20-min intervals from 09:00 h through to 09:00 h the following day.

Steroid analysis

Serum steroids (17OHP, A4, testosterone) in the serum samples from the phase 3 study were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS; Q2 Solutions, USA). Serum 17OHP, A4, and testosterone were measured in the healthy controls by LC-MS/MS as previously described.[13] Salivary steroids (17OHP, A4, testosterone, 11OHA4, 11 KT) were measured by LC-MS/MS as previously described.[14]

Statistical analysis

Data analysis was carried out in *R: A Language and environment for statistical computing* (R Foundation for Statistical Computing, Vienna, Austria), packages employed listed in Appendix pp 3. Missing profile measurements were interpolated between points within each profile or imputed with the adjacent value if the missing point was at the beginning or end of the profile. Profiles were modelled using a cubic smoothing spline function that calculated the area under the curve (AUC). Natural log (ln) transformations were carried out to achieve normality of skewed variables. Bivariate linear regression was used to compare levels of ln transformed biomarkers with degree of correlation quantified by R^2, the square of Pearson's correlation coefficient. Linear mixed effects models with sine and cosine terms were

	Healthy participants	All patients with CAH	Patients with CAH on standard glucocorticoid replacement[a]	Patients with CAH on chronocort
Number of participants	20 Male = 13, Female = 7	122[b] Male = 44, Female = 78	61 Male = 25, Female = 36	61 Male = 19, Female = 42
Age: mean years (standard deviation)	28.4 (12.3)	36.3 (11.6)	37.5 (12.8)	35.2 (10.3)
Number of 24-h profiles	20[c]	477	301	176
Number of readings	1440[d]	6202	3914	2288
Serum 17OHP (nmol/l) from 2-hourly sampling over 24 h				
Mean	3.7	33.7	43.1	17.5
Standard deviation	3.0	79.5	93.9	40.7
Median	2.7	5.1	6.7	3.5
Quartile 1 to quartile 3	1.5 to 4.9	1.8 to 24.3	2.2 to 33.6	1.2 to 11.9
Amplitude of cosinor mixed-effects model[e]	0.44 (95% CI: 0.33 to 0.54)	–	7.73 (95% CI: 7.67 to 7.79)	0.83 (95% CI: 0.77 to 0.90)
Serum androstenedione (nmol/l) from 2-hourly sampling over 24 h				
Mean	4.0	3.6	4.4	2.4
Standard deviation	3.6	6.1	7.1	3.4
Median	3.0	1.5	1.7	1.4
Quartile 1 to quartile 3	2.1 to 4.6	0.7 to 3.5	0.7 to 4.4	0.6 to 2.7
Amplitude of cosinor mixed-effects model[e]	0.71 (95% CI: 0.64 to 0.78)	–	0.50 (95% CI: 0.47 to 0.54)	0.03 (95% CI: 0.01 to 0.05)

[a]Summary statistics for biomarker readings are calculated including readings from patients that were later randomised to take Chronocort, but at the time of their first profile, were still taking standard glucocorticoid replacement. [b]117/122 patients completed all four study visits. [c]1/20 patients only had profiling data available for Androstenedione, and not 17OHP. [d]1368 values available for 17OHP as one patient only had profiling of Androstenedione conducted. [e]Full cosinor model parameters in Supplementary Table S3 linear mixed effects model. Confidence intervals estimated by calculating across 1000 bootstrap replications.

Table 1: **Participant demographics and summary statistics of serum 17-hydroxyprogesterone (17OHP) and androstenedione (A4).**

employed to quantify the circadian rhythm of markers. Bayesian multiple change point analysis was employed on ln transformed values to assess the hypothesis that 17OHP is likely to increase more rapidly at higher concentrations of androstenedione due to 17, 20 lyase saturation. Change point models were assessed by $\hat{R}$, a statistic indicating a suitable representation of the data at values between 1.0 and 1.1.[15]

Ethics

The study protocols for the phase 3 study were approved by North West - Liverpool Central Research Ethics Committee (15/NW/0868), Institutional Review Boards and the Medicines and Healthcare Products Regulatory Agency (NCT02716818, Eudract 2015-000711-40). Written informed consent was obtained from all participants. The trials were performed in accordance with the principles of the Declaration of Helsinki.

Role of funders

The original study on MHRC was funded by Diurnal ltd. a phase 3 clinical study to test the efficacy of Chronocort, results used to licence the drug Chronocort. The funders played no role in the further data analysis, interpretation, or writing contained within this manuscript.

Results

Serum 17OHP and A4

The 24-h absolute levels of serum 17OHP in nmol/l overlaid on A4 10× nmol/l (to allow comparison as in CAH absolute levels of A4 are ~10× lower than 17OHP) showed similar profiles, with changes in 17OHP and A4 paralleling each other in the patients with CAH (Fig. 1). The same was true for the profiles from healthy volunteers although the absolute levels of 17OHP and A4 were of the same magnitude and generally lower (Table 1, 1/20 participants missing profile data for 17OHP). In healthy participants, there was a low amplitude of 17OHP (0.4 nmol/l) and Androstenedione (0.7 nmol/l) (Appendix pp 4–5). In contrast, patients with CAH had large variability in serum 17OHP levels exhibiting a diurnal rhythm with an amplitude of 7.7 nmol/l and peak at 07:54 h in those taking standard glucocorticoid replacement. During the trial, control improved in both arms but more markedly in the MRHC arm and the amplitude of the 17OHP rhythm reduced to 0.8 nmol/l in those on MRHC. Similar results with lower absolute levels were seen for A4.

Relationship between serum 17OHP and A4 levels

In both healthy participants and patients with CAH, there was no lag on cross-correlation of 17OHP with A4 confirming that they change in parallel (Fig. 2). In healthy participants, mean 24-h 17OHP and A4 levels were very similar, whereas in patients with CAH 17OHP levels were generally much greater than those of A4 (Table 1). In patients with CAH, Bayesian multiple change point analysis assessing ln17OHP on lnA4 converged on a change point of 17OHP at 4.5 nmol/l (149 ng/dl) (n = 6202, 95%, credible interval:4.0–5.0 nmol/l, $\hat{R}$ = 1.007) (Fig. 3, Appendix pp 6–7). Below the change

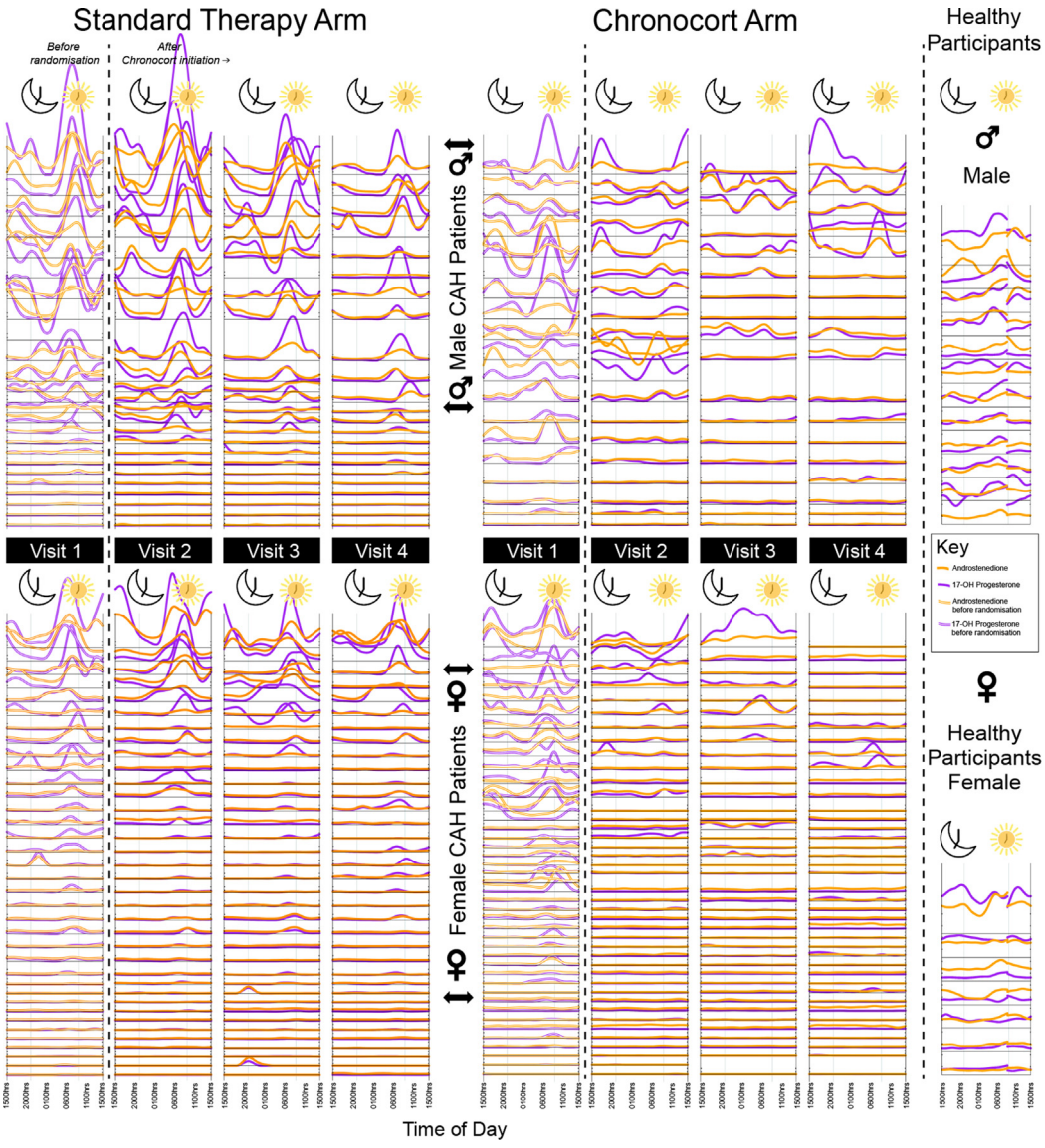

***Fig. 1:* 24-h profiles of serum biomarkers.** Profiles of 17-OH progesterone (17OHP) and androstenedione (A4) in healthy participants and patients with congenital adrenal hyperplasia (CAH) plotted to show the relationship between 17OHP and A4 within individuals. For patients with CAH 17OHP in nmol/l is overlaid on A4 10× nmol/l to allow comparison as in CAH absolute levels of A4 are ~10× lower than 17OHP whereas in healthy participants they have similar absolute levels of 17OHP and A4.

point, there was a greater decrease in A4 for a similar decrease in 17OHP. Whilst women tended to have lower biomarkers than men, the relationship between biomarkers or change point when modelled separately did not differ between sexes, or whether on MRHC versus conventional, immediate-release glucocorticoid. The relationship between serum 17OHP and A4 was different for healthy participants who, for similar levels of 17OHP, had higher levels of A4 than patients with CAH. Thus in patients with CAH, when the A4 was at the ULN, the 17OHP was elevated, and when the 17OHP was in the normal range, the A4 levels were suppressed: a 17OHP <38 nmol/l (1250 ng/dl) was associated with an A4 in the normal range <5 nmol/l (143 ng/dl) in 95% of patients.

Relationship between serum 17OHP, A4 and testosterone

In healthy women, testosterone levels were higher for the same level of 17OHP than in women with CAH, whereas for A4, the relationship between testosterone and A4 was similar for healthy women as in those with CAH (Appendix pp 9–10). In male patients, there was a

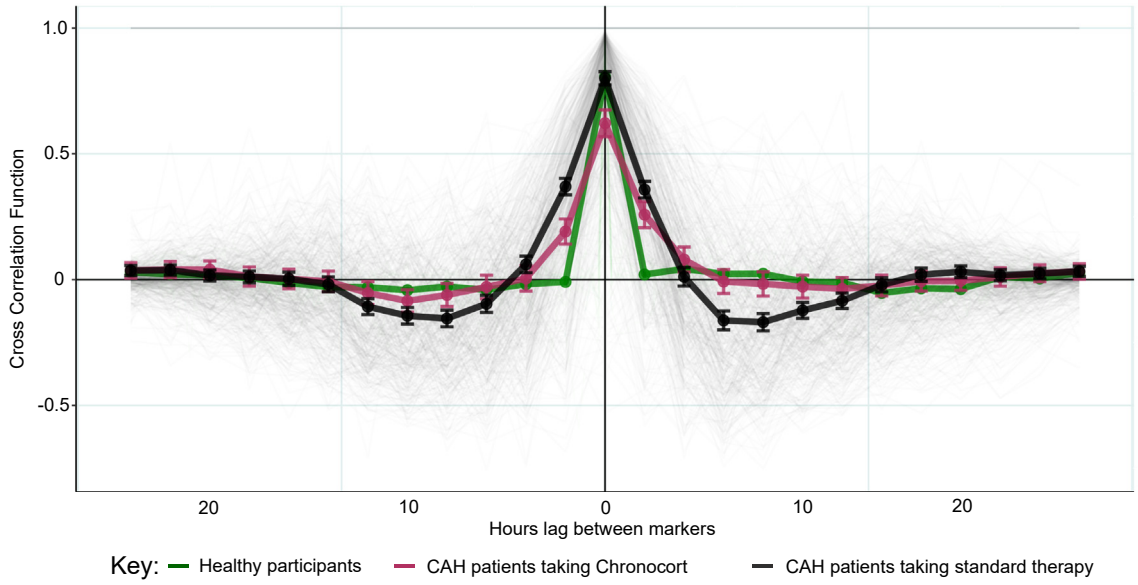

***Fig. 2:* Cross-correlation of serum 17-OH progesterone and androstenedione by group.** Cross-correlation plot of 17-OH progesterone (17OHP) and Androstenedione (A4) in healthy participants and patients with congenital adrenal hyperplasia (CAH) showing there is no lag between markers, with peak at zero demonstrating that the markers change in parallel over time. Points show mean of the estimate within groups, error bars show standard error of the mean, grey lines in background show individual patient cross-correlation plots.

very weak negative correlation between 17OHP, A4 and testosterone (17OHP: n = 169, R^2 = 0.021, p = 0.10, A4: n = 169, R^2 = 0.033, p = 0.02).

Salivary profiles of 17OHP, A4, 11OHA4, testosterone, and 11 ketotestosterone

Absolute profiles of all five salivary steroids measured overlaid on serum 17OHP and A4 profiles appropriately scaled showed similar patterns in patients with CAH. Correlation of each salivary marker was strong within female patients with CAH, ranging from r = 0.7 to 0.9 for each of the five salivary steroids with both serum 17OHP and A4. Correlations were weaker in male patients with CAH, driven partly by a lower sample size (Fig. 4, Appendix pp 11–12).

Dose of glucocorticoid replacement and disease control

There was a wide variation in total daily replacement glucocorticoid dose across the cohort from 10 mg to 80 mg hydrocortisone equivalent. Overall mean (SD) total daily hydrocortisone equivalent at time of profile measurement was 28.8 mg (10.6) mg and was similar in those taking standard glucocorticoid therapy 28.9 (10.9) mg and those taking MRHC 28.8 (10.0) mg. Comparing AUC of serum 17OHP and A4 within patients on the same dose at two different visits, we saw similar control between the two visits (men: n = 93, R^2 = 0.79, 0.90, women: n = 218, R^2 = 0.44, 0.55). The distribution of serum 17OHP was similar across the dose range (n = 477, slope = –0.007, standard error of the mean (SEM) = 0.008, p = 0.34) (Appendix pp 13–16), with 187/477 profiles measured within the range of serum 17OHP AUC seen in healthy controls. For serum 17OHP the distribution above and below the healthy controls was similar (140 above, 150 below), whereas for A4 AUC, the majority had an AUC below that of healthy controls (none above, 357 below).

Discussion

We investigated the relationship between adrenal steroid hormones in healthy participants and patients with CAH to better understand the biochemical monitoring of CAH. Serum 17OHP and A4 showed similar changes over time in both healthy participants and patients with CAH but absolute levels and the relationship between 17OHP and A4 differed between healthy participants and patients with CAH having a proportionally lower A4 for the same level of 17OHP. Salivary 17OHP and A4 showed similar changes and correlated with salivary testosterone and 11-ketotestosterone in women. Measurements of 17OHP and A4 made on different days whilst receiving the same glucocorticoid dose were similar. Patients on MRHC had flatter profiles, similar to healthy participants, such that a single measurement of either 17OHP or A4 reflected the overall 24-h levels. In contrast, patients on conventional, immediate-release glucocorticoid treatment generally had a diurnal rhythm with an increased amplitude of both 17OHP and A4 in the morning.

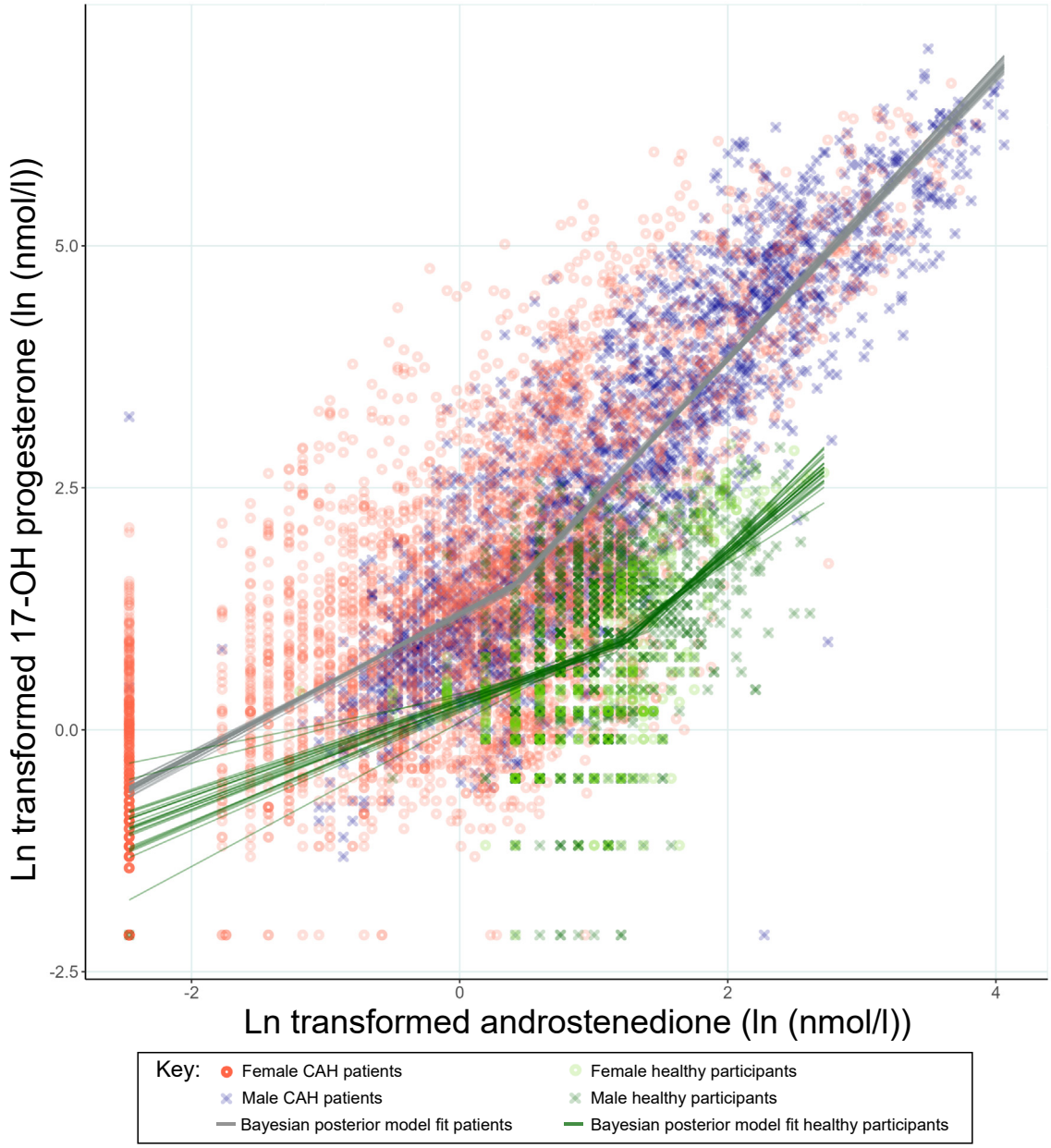

***Fig. 3:* Changepoint analysis of 17-OH progesterone on androstenedione.** Regression of ln transformed area under curve 17-OH progesterone (17OHP) on androstenedione (A4) in healthy participants and patients with congenital adrenal hyperplasia (CAH). In healthy participants there are higher level of A4 for same levels of 17OHP. Bayesian changepoint analysis of 17OHP on A4 converged on changepoint in patients with CAH at 4.5 nmol/l 17OHP (149 ng/dl) ($\hat{R} < 1.008$), compared to changepoint in healthy participants at 2.4 nmol/l (80.0 ng/dl) ($\hat{R} < 1.020$). Grey lines show 25 randomly selected Bayesian posterior model fits close together, thus showing stable convergence of the model.

The 24-h serum profiles of 17OHP and A4 showed parallel changes in both healthy participants and patients with CAH, with no lag on cross-correlation. This means 17OHP and A4 have a similar serum half-life and provide similar information about androgen control over time. In healthy participants, profiles of serum 17OHP and A4 were relatively flat with low amplitude (<1 nmol/l), suggesting no clinically significant circadian rhythm. At baseline, most patients with CAH on conventional, immediate-release glucocorticoid treatment had a diurnal rhythm of both markers, with a rise over the early hours peaking in the morning consistent with the fact that standard glucocorticoid replacement cannot control the overnight rise in ACTH. This has led

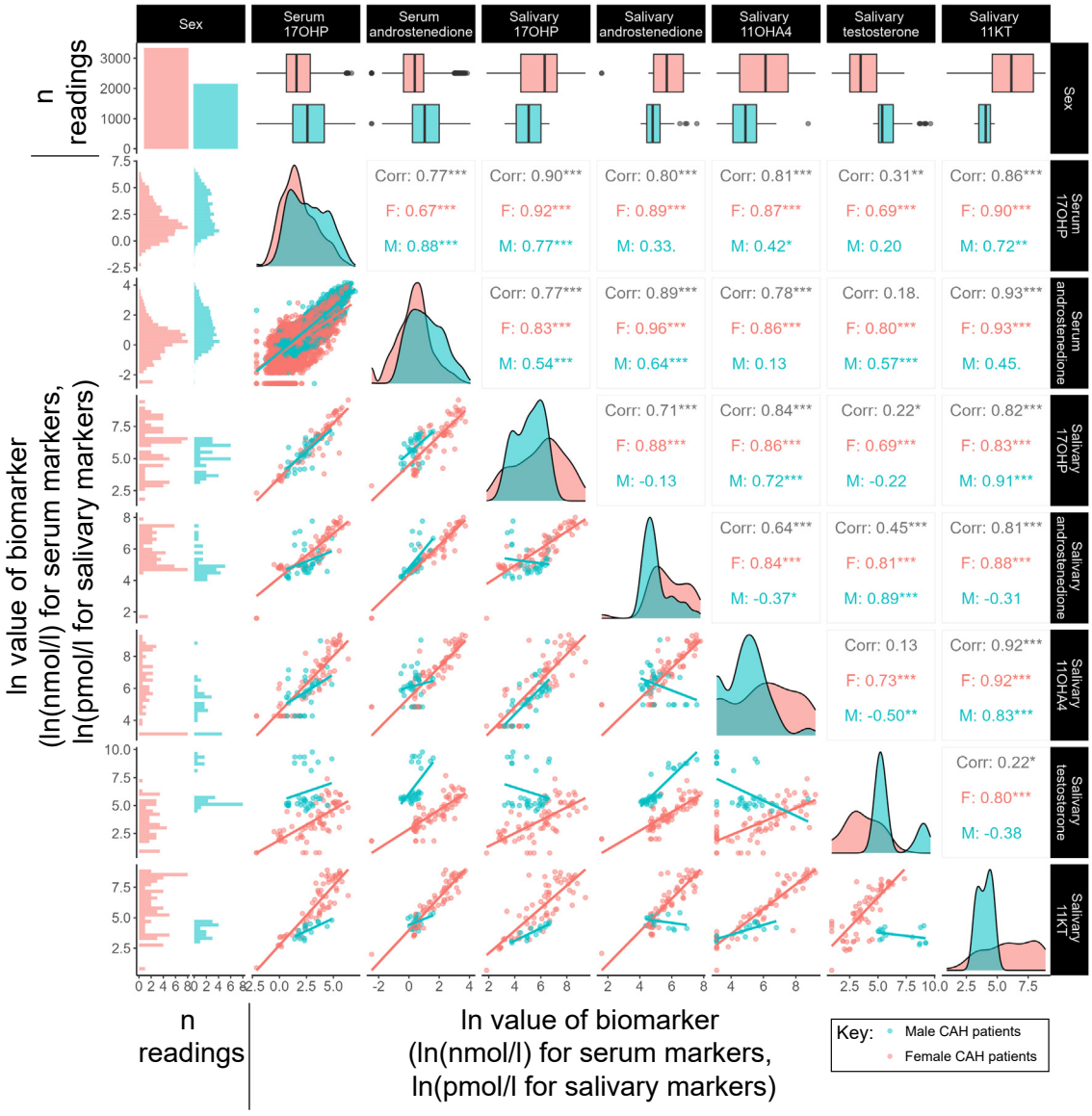

Fig. 4: **Matrix correlation of serum and salivary steroids.** Matrix of Correlations between serum 17-OH progesterone (17OHP), androstenedione (A4) and salivary 17OHP, A4, 11-OH androstenedione, Testosterone and 11-ketotestosterone. Point measurements ln transformed measured at the same time of day. Saliva measured within 9 patients across 3 visits at one study centre.

clinicians to measure steroid profiles on standard treatment and to debate whether to take measurements before or after the morning glucocorticoid dose, as a single measurement does not reflect the overall 24-h biochemical control.[6] In contrast, patients on MRHC had flatter profiles (amplitude <1 nmol/l for 17OHP), similar to healthy participants. This demonstrates that MRHC treatment can be monitored in most patients by taking a sample at any time in the daytime and independent of the time of MRHC intake as the result will reflect the 24-h levels. MRHC is a modified-release formulation of hydrocortisone with delayed release such that a nighttime dose provides a physiological cortisol rise overnight to peak in the morning with a second dose taken in the morning to fill in the diurnal rhythm. Thus, monitoring on MRHC doesn't need to be done before or after dosing but can be done any time in a morning or afternoon clinic, and a single measurement will generally reflect control over the 24 h, as 17OHP and A4 levels do not show a diurnal rhythm on MRHC.

The relationship between serum 17OHP and A4 differed between healthy participants and patients with CAH. In healthy participants, absolute levels of 17OHP

and A4 were similar but patients with CAH had proportionally much lower A4 levels for the same level of 17OHP. In addition, our results showed a change point in the relationship between 17OHP and A4 in patients with CAH whereby when 17OHP is in the reference range A4 levels were proportionally even lower. This can be explained by the pathophysiology of CAH, where 17OHP accumulates prior to the 21-hydroxylase enzyme block. The excess levels of 17OHP result in the atypical conversion of 17OHP to A4 by CYP17A1 17,20-lyase activity, which physiologically has a much higher preference for the conversion of 17OH-pregnenolone to DHEA, which in CAH appears suppressed. The excess 17OHP drives increased androgen production through A4, which also feeds the 11-oxygenated androgen pathway. Thus, when 17OHP levels are brought into the normal range, A4 is neither generated from 17OHP nor DHEA. The relevance of this to monitoring patients with CAH receiving MRHC treatment is that elevated A4 levels, even in the upper half of the reference range, reflect poor control of androgens and, conversely, low A4 does not necessarily reflect glucocorticoid overtreatment. This is evident from our study where a proportion of patients on total daily doses of MRHC 10–15 mg/day still had very low A4 levels in whom one would be hesitant to reduce such a low daily dose any further to avoid giving inadequate hydrocortisone replacement for adrenal insufficiency. It is unclear why androgen biosynthesis does not reset itself once better disease control is achieved. One hypothesis could be that there is an intra-adrenal feedback loop, with persistent androgen excess resulting in downregulation of CYP17A1 or specifically CYP17A1 17,20 lyase activity. Future research will need to investigate whether the relationship between 17OHP and A4 normalises as treatment improves.

The salivary steroid profiles of 17OHP and A4 correlated well with the serum profiles suggesting that where salivary measurements are available, they could be used instead of serum measurements. This is consistent with the previously published data of single measurements made in the day in a large cohort of children with CAH and healthy controls.[16] This has the advantage that salivary steroids are stable at room temperature and can be collected at home and sent to the laboratory, avoiding the need for venipuncture. In women, the salivary testosterone, 11OHA4 and 11-ketotestosterone all strongly correlated with the serum and salivary 17OHP and A4, suggesting that controlling 17OHP and A4 also controls the other androgen pathways in the adrenal gland as has previously been shown with urine analysis.[17] As the correlation between the 11-oxygenated androgens and serum and saliva 17OHP and A4 was so good, it is not clear whether they will add anything in assessing control in clinical practice, although this is based on a limited data set.

The dose of glucocorticoid given either as standard treatment or MRHC did not correlate with control of 17OHP across patients, despite improved control in individual patients with increasing the glucocorticoid dose. All the patients studied had proven established classic CAH but there was a wide spectrum of total daily hydrocortisone equivalent dose from 5 mg to 80 mg, with the spectrum of control similar at each dose. Compliance was good in the study which was closely monitored. Half of the patients (50.3%) were taking either standard treatment or MRHC with hydrocortisone equivalent doses above that recommended from adult adrenal replacement (25 mg/day). It is not clear what factors apart from glucocorticoid dose and circadian delivery of hydrocortisone determine the level of control, and this is something to be addressed by future research. However, we have noted that with improved control on MRHC the dose of hydrocortisone can be reduced over time suggesting length of time with poor maybe a variable important in control due to hyperplastic adrenals.[11]

Current guidelines recommend that 17OHP should not be titrated into the reference range as this risks overtreatment with glucocorticoid; however, no recommendation on the absolute level is given.[9] Cohort studies have suggested that "optimal control" would be a 17OHP <36 nmol/l (1200 ng/dl), which is below 3–4x the ULN.[18,19] Our analysis showed that a 17OHP <38 nmol/l (1250 ng/dl) was associated with an A4 in the normal range <5 nmol/l (143 ng/dl) in 95% of patients. This level of 17OHP is similar to that used in the cohort studies to define optimal control and was used in the clinical trial. However, the absolute level of control of adrenal androgens in CAH depends on the clinical goals for the patients, often dependent on age and sex. During childhood, optimising growth and preserving fertility are important targets in the management of CAH. In young adults, fertility becomes increasingly important, and in older adults avoiding iatrogenic glucocorticoid excess becomes important. Among the patients on MRHC in the clinical trial, a number of female patients became pregnant, as well as the female partners of male patients, suggesting that controlling the adrenal androgens throughout the 24 h is important for fertility.[11] Our results show that if the 17OHP is controlled within 3–4× ULN, then the A4 will be in the reference range or low. Based on the close relationship of A4 to testosterone and 11-ketotestosterone in women, elevated A4 levels are likely to be associated with virilisation and infertility. Therefore, in women prioritising fertility treatment, titrating A4 into the lower half of the reference range is likely to be more successful; in parallel to A4, 17OHP and progesterone which typically accumulate in CAH will normalise, supporting improved implantation and early pregnancy outcomes. The absolute level of 17OHP and A4 used to titrate the hydrocortisone dose should depend upon the goals of

treatment and the patient's current dose and treatment regimen. More long-term studies accounting appropriately for age and sex are required to determine optimal control for growth and fertility. Accepting that 17OHP is higher than A4, both measurements give a similar reflection of control and a 17OHP <38 nmol/l and an A4 of <5 nmol/l reflect reasonable control for most patients.

The limitations of this study include the fact that not all steroid measurements were made in all participants and that serum steroid measurements were carried out by different assays in study participants and healthy controls, albeit both were established and validated LC-MS/MS assays. The number of healthy participants was lower that CAH patients and the sex ratio different. We have used ordinary least squares regression to describe relationships between biomarkers despite multiple measurements within patients and relationships that are likely non-linear for the sake of simplicity. Relatively few patients had 11-oxygenated androgens measured but these were detailed profiles and the largest dataset of its kind. The density of data with 2-hourly 24-h profiles being repeated 4 times in a cohort of over 100 patients with CAH provides a robust reference set against which comparisons can be made. Most patients in this study were salt wasting and data was not available to investigate whether the relationship between 17OHP and A4 differs between genotypes. However, although there is an overall good genotype–phenotype correlation for the salt wasting phenotype there is not an association between biochemical control and genotype.[20] The patients in this study were adults with CAH and therefore we can't assume the same relationship applies between 17OHP and A4 in children and young people.

In conclusion, measuring either 17OHP and or A4 gives similar information about the control of adrenal androgens; however, clinicians should be aware that in patients with CAH A4 levels are proportionally lower than 17OHP, so where tight control is required, such as a woman wishing to be pregnant, A4 levels may be very low, and the hydrocortisone dose should not be reduced below a typical adrenal replacement dose. On MRHC, a single measurement of either 17OHP and/or A4 can be taken in the morning or early afternoon, independent of the time of drug dosing, and used to monitor treatment. Either serum or saliva measurements can be used as the levels parallel each other.

Contributors

R.R., D.M., W.A. and J.N-P. conceptualised the study and developed the methodology. R.R. and D.P.M. acquired funding. A.P., E.B., L.S., A.T., A.P., A.H., A.J., D.M., J.N-P., A.R., N.R., M.S., P.T., B.K., W.A. and R.R. provided resources and project administration to carry out the study. G.S.C, J.D., Z.L., N.K. and R.R provided supervision and support with software, visualisation and formal analysis. N.L. and R.R. formally accessed, verified and curated the underlying data. N.L. wrote the original draft, with all co-authors supporting the writing with review, editing and approval of the final manuscript.

Data sharing statement

Original study protocol and statistical analysis plan can be found: https://clinicaltrials.gov/study/NCT02716818.

Datasets generated during and analysed during the current study are not publicly available but are available from the corresponding author on reasonable request. The code used to model data within R to carry out this research can be found: https://github.com/neilklawrence/chronocort_modelling.

Declaration of interests

A.P., E.B., D.P.M., J.N-P., A.R., N.K. and R.R. report additional COIs: R.R. is a Director of Diurnal Group Plc. A.P. receives unrelated research funds from Diurnal Limited and HRA Pharma. D.A.R. has received honoraria from Neurocrine biosciences. N.R. has received honoraria from Lundbeck, Crinetics, Spruce Biosciences and Neurocrine Biosciences. J.N-P. has received unrelated research funds from Diurnal Group Plc and Crinetics. D.M. received unrelated research funds from Diurnal Limited, Neurocrine Biosciences and Adrenas Therapeutics, all through the National Institutes of Health Cooperative Research and Development Agreements. N.K. has received research funds from Neurocrine Biosciences. G.C. is a NIHR Senior Investigator. A.P. delivered results for this study through the NIHR Birmingham Biomedical Research Centre. The views expressed in this article are those of the author(s) and not necessarily those of the NIHR, or the Department of Health and Social Care.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2025.105749.

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