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Re-setting the abnormal circadian cortisol rhythm in adrenal incidentaloma patients with mild autonomous cortisol secretion

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Disclosure statement

RC and JLA were HRA PHARMA employees at the time of study conduct and analysis. CG is an HRA PHARMA employee. JNP has received research funding and honoraria from HRA Pharma.

Summary

Context: Adrenal incidentalomas (AI) are found commonly on axial imaging. Around 30% exhibit autonomous cortisol secretion (AI/ACS) associated with increased cardiovascular events and death.

Objective: We hypothesised that AI/ACS patients have an abnormal cortisol rhythm that could be reversed by use of carefully timed short-acting cortisol synthesis blockade, with improvement in cardiovascular disease markers.

Design, setting and participants: In a phase 1/2a, prospective study (Eudract No 2012-002586-35) we recruited 6 patients with AI/ACS and two control groups of 6 sex, age, BMI-matched individuals: (i) patients with adrenal incidentalomas and no ACS (AI/NoACS) and (ii) healthy volunteers with no adrenal incidentaloma (HC). 24-hour circadian cortisol analysis was performed to determine any differences between groups and timing of intervention for cortisol-lowering using the 11β-hydroxylase inhibitor, metyrapone. Circadian profiles of serum IL-6 were assessed.

Results Serum cortisol levels in Group AI/ACS were significantly higher than both Group AI/NoACS and Group HC from 18:00h-22:00h (AUC difference:0.81nmol/L.h;p=0.01) and from 22:00h-02:00h (AUCdifference:0.86nmol/L.h;p<0.001). In light of these findings patients with ACS received metyrapone 500mg at 18:00h and 250mg at 22:00h and cortisol rhythms re-assessed. Post-intervention evening serum cortisol was lowered, similar to controls [18:00h–22:00h (AUC difference:-0.06nmol/L.h;p=0.85); 22:00h-02:00h (AUC difference:0.10nmol/L.h;p=0.76)]. Salivary cortisone showed analogous changes. IL-6 levels were elevated pre-treatment [22:00h-14:00h AUC difference 0.42pg/mL.h; p=0.01] and normalized post-treatment.

Conclusions: In AI/ACS the evening and nocturnal cortisol exposure is increased. Use of timed evening doses of metyrapone re-sets the cortisol rhythm to normal. This novel treatment paradigm is associated with a reduction in the cardiovascular risk marker IL-6.

Introduction

Patients with adrenal incidentalomas (AI) and low-grade excess cortisol secretion have higher mortality due to cardiovascular events and infections (1, 2). The prevalence of AI is <1% of those aged 20y, but increases to around 10% of those aged 70y (3). Between 30-50% of AI exhibit low-grade cortisol secretion variously termed "subclinical Cushing's" or "subclinical hypercortisolism" or more recently "autonomous cortisol secretion (ACS)" (4, 5), since patients lack the classical clinical features of Cushing's syndrome (6, 7). Nevertheless, patients with AI/ACS have more cardiovascular events, osteoporosis and fractures, and incident diabetes(1, 2, 8-10),(11). Together these data strongly support that AI/ACS are detrimental to health, and that this is a common problem.

A blunted cortisol circadian rhythm, with higher evening and night levels, is a sensitive indicator of overt Cushing's syndrome (6, 12). In AI, however, the cortisol rhythm has never been systematically studied in fine detail by measuring hourly cortisol levels. Normally, cortisol concentrations reach a nadir around midnight, and rise at around 02:00h to 04:00h, peaking 30 to 60 minutes after waking and then gradually decline towards the quiescent phase in the early evening. In overt Cushing's inappropriately high cortisol levels result in multiple complications, including impaired glucose tolerance and diabetes, visceral obesity, psychiatric illness, metabolic bone and cardiovascular disease, infections and increased mortality (6, 13). Interleukin-6 (IL-6) is high in patients with Cushing's syndrome(14, 15) and this cytokine is associated with endothelial dysfunction and implicated in the pathogenesis of atherosclerosis (16); a recognised complication of AI/ACS (17).

We hypothesised, therefore, that patients with AI/ACS, as identified using the 1mg dexamethasone suppression test (ONDST), have higher evening cortisol concentrations due to autonomous secretion from the adrenal, with this being associated with a state of low-grade inflammation, as indicated by IL-6 levels. We have assessed the baseline cortisol and IL-6 rhythms in these patients and then tested whether administration of a short-acting 11β-hydroxylase inhibitor, metyrapone, at specific

time points could 'reset' the cortisol rhythm in patients, and what impact this would have acutely on IL-6 levels.

Methods

Study Design and patients

This was a phase 1/2a prospective, open-label, controlled, single centre study carried out in the NIHR Clinical Research Facility, Sheffield Teaching Hospitals NHS Foundation Trust, UK in patients diagnosed with AI (Figure 1). Six patients (Group AI/ACS) with unilateral or bilateral AI showing benign characteristics on a CT-scan (precontrast <10 Hounsfield Units) or magnetic resonance imaging (MRI) together with an ONDST serum cortisol >80nmol/L, or 60-80nmol/L with an ACTH <2.2pmol/L (10pg/mL), and no features of clinical Cushing's syndrome were recruited. Baseline characteristics were similar to the target population of interest (Supplementary Table 1)(8). Two control groups of 6 sex-, BMI- and age-matched subjects were recruited who had an ONDST serum cortisol <50nmol/L: i) no AI - AI/NoACS or ii) normal adrenal glands on abdominal MRI (Group Healthy Controls (HC). Inclusion criteria were: 45 to 80 year old males and post-menopausal females, and stable antihypertensive and diabetic medications for 4 weeks prior to screening. Exclusion criteria: patients with clinical features associated with overt Cushing syndrome, history of malignancy, alcohol dependence or drug abuse, primary adrenocortical insufficiency, severe uncontrolled diabetes (fasting plasma glucose >15.0mmol/L or glycated haemoglobin >9% [75mmol/mol]), severe uncontrolled hypertension (>190/120 mmHg), severe liver disease, renal impairment (serum creatinine ≥120 μmol/L), clinically significantly impaired cardiovascular function, uncontrolled severe active infection, night-shift workers, patients with depression or psychosis, treatment with glucocorticoids (oral, spray or cream) in the last 3 months, concomitant treatment with any other drug known to affect the HPA axis, CBG or the CYP450 3A4 cytochrome system and adrenocortical tumours >4 cm (Supplementary Figure 1).

The study protocol was approved by the East Leeds National Research Ethics Service committee, and the Medicines and Health Regulatory Authority, UK. Written informed consent was obtained from all participants. The study was registered with the European Clinical Trials Database (Eudract No. 2012-002586-35), and is reported according to the Transparent Reporting of Evaluations with Nonrandomized Designs (TREND) [16].

Baseline study

For study visits participants arrived at the clinical research facility around two hours before study start (~16:00h), for a 24-hour sampling period. An intravenous cannula was inserted with a three way tap for sampling via long extension line to avoid disturbing sleep and kept patent by slow intravenous 0.9% normal saline. Dead space volume was discarded at each sampling time point prior to sample collection. Standard meals were provided at 18:00h, 07:30h and 12:30h. The study was carried out under stable, environmental conditions and lights were turned off at 23:00h. Hourly blood samples (Day 1 18:00h to Day 2 18:00h) and hourly salivary cortisol/cortisone during waking hours between 18:00h and 23:00h and between 08:00h and 18:00h were collected. Patients were not allowed to eat, drink and wash their teeth for 30 minutes before salivary tests. Full blood count, liver function tests, urea, creatinine and electrolytes, fasting blood glucose and lipids, and 08:00h ACTH were also measured.

Interventional study

By a priori design (Figure 1) an interim analysis was performed on all baseline data to assess if there were differences in the cortisol rhythms between groups. These data were used to determine the timing of administration of metyrapone. Based on these analyses Group AI/ACS were administered 500 mg of metyrapone at 18:00h after a standard meal. Hourly serum cortisol and salivary cortisol/cortisone (measured during waking hours only) levels were measured. These data were then analysed and on the basis of these results a subsequent intervention was performed with

metyrapone 500 mg at 18:00h after a standard meal and 250 mg at 22:00h with a snack in patients (Group AI/ACS). Similar investigations to those carried out during the baseline visit were performed during these interventions.

Assays

Serum and salivary cortisol and salivary cortisone were measured by LC-MS/MS, as previously described (18). Serum IL-6 was measured by a bead-based immunoassay and the limit of detection was 1.6 pg/ml (19). The normal upper limit of IL-6 is 4pg/mL (20).The intra-assay CV was 3% at 35.6 pg/mL and the inter-assay CV was 6% at 38.8 pg/mL. Plasma ACTH was measured in a Siemens Immulite 2000 chemiluminescent assay: analytical range 1.1 – 275pmol/L; interassay CVs 6.1% at 7.5pmol/L and 4.3% at 100pmol/L. DHEA was measured by LC-MS/MS.

Statistical Analysis

Data are summarised per group and time points by descriptive statistics using number of patients [N], mean, SD for continuous variables and absolute counts and relative frequencies (n and %) for categorical data. Pharmacokinetic (PK) analyses were performed using WinNonlin Professional V5.3 Software, Certara USA, Princeton, NJ, USA and Matlab Version 8.2 (Mathworks, Natick, MA, USA). Concentration time profiles were designed for serum cortisol, salivary cortisone and serum IL-6 at baseline and post-intervention (metyrapone). As salivary cortisone has been shown to be a superior surrogate marker for serum cortisol when compared to salivary cortisol our analysis is based on salivary cortisone measurements (18). The primary endpoint for the analysis was the geometric 4-hourly AUCs over 24 hours starting from 18:00h. Wilcoxon signed rank test was used for paired sample analysis. Where samples were unpaired the differences and p-values are computed using bootstrapped (N = 50,000) Welch's t-test. AUC was computed from $\log_{10}$ transformed data. Missing values were linearly interpolated. The full 24-hour profile was also assessed for salivary cortisone.

Missing values, (night-time & sporadic) are imputed by applying the inverse of our published LME (linear mixed-effects) model to Serum Cortisol values (18). Similar AUCs were measured for IL-6.

For 24-hour AUCs independent sample t-test was used for normally distributed data and Mann Whitney test for non-normally distributed data. Other secondary endpoints were the peak (C_{max}), time of peak (T_{max}) from midnight, trough (C_{min}) and relative amplitude (absolute amplitude [50% of the difference between the level attained at C_{max} and the level attained at C_{min}] expressed as a percentage of the 24-h mean cortisol). ANOVA (normally distributed) with LSD as post-hoc test or Kruskal-Wallis tests (not normally distributed) were used to assess for differences in PK parameters between groups.

Results

A total of 20 patients were included in the study: 6 healthy subjects, 6 patients with AI/NoACS and 8 patients with AI/ACS. Two AI/ACS patients were withdrawn from the study after the first intervention: one for an unrelated SAE (prostate adenocarcinoma, diagnosed during the study); the other because of work-related time commitment reasons. Two additional AI/ACS patients were included for the subsequent intervention with metyrapone 500 mg at 18:00h and 250 mg at 22:00h. Baseline data for Group AI/AS taken into account in the final analysis are from the 6 patients who concluded the study.

There were no differences in sex, mean (SD) age [65 (5.8) vs 65 (3.4) vs 65 (4.9) years; p = 0.9] and BMI [31 (6.9) vs 30 (5.1) vs 29 (8.9) kg/m^2; p = 0.6], between groups (Supplementary Table 1). In the AI/ACS group ONDST serum cortisol varied between 59 nmol/L and 127 nmol/L; the highest ONDST serum cortisol in the other groups was 31nmol/L (Supplementary Table 1).

Serum cortisol rhythm at baseline

Analysis of the baseline 24-hour serum cortisol rhythm showed that there were no differences in AUCs between AI/NoACS and HC throughout the entire 24-hour period (p = 0.33). In contrast levels in AI/ACS were significantly higher than in these groups only between 18:00h and 22:00h (AUC difference: 0.81 nmol/L.h; p = 0.01) and between 22:00h and 02:00h (AUC difference: 0.86 nmol/L.h; p <0.001) (Figure 2a).

The mean (SD) relative amplitude in the AI/ACS group at 85% (21) was significantly lower than either Group AI/NoACS 144% (28) and Group HC 120% (19) (p = 0.002), whilst, C_{min} was significantly higher in Group AI/ACS 94 (45)nmol/L compared to Group AI/NoACS 53 (16)nmol/L and Group HC 44 (19)nmol/L (p = 0.03), but C_{max} (p = 0.18) and T_{max} (p = 0.84) were similar in all groups. These data demonstrate blunting of the physiological cortisol rhythm in patients with AI/SCS due to higher minimum evening values.

Serum cortisol rhythm post intervention

In view of the identified serum cortisol differences, the sampling study was repeated in patients with AI/ACS with administration of metyrapone 500 mg at 18:00h to assess if this could 'reset' the rhythm to normal. There was a significant reduction in log-converted $AUC_{18\text{-}22}$ (p = 0.03) but not in $AUC_{22\text{-}02}$ (p = 0.17), resulting in serum cortisol levels similar to controls in the four hour period after 18:00h (AUC difference -0.40 nmol/L.h; p = 0.93), but with values then rising towards baseline after 22:00h. In view of this, sampling was repeated in AI/ACS patients with metyrapone 500mg at 1800h and 250mg at 22:00h, resulting in no differences between Group AI/ACS and controls in: log-transformed AUC between 18:00h and 22:00h (AUC difference: -0.06 nmol/L.h; p = 0.85) and between 22:00h and 02:00h (AUC difference: 0.10 nmol/L.h; p = 0.76), in serum cortisol levels throughout the 24-hour period (p = 0.29) and in mean (SD) relative amplitude [117 (39)%; p = 0.27], C_{min} [56 (26)nmol/L; p = 0.62], C_{max} [442 (108)nmol/L; p = 0.21] and T_{max} [9 (2.5)h; p = 0.80). These data indicate that cortisol exposure in Group AI/ACS had been normalised by 're-setting' the cortisol rhythm (Figure 2b).

Salivary cortisone and cortisol at baseline

Salivary cortisone accurately reflects total and free serum cortisol levels (18, 21). Log-transformed $AUC_{20:00–23:00}$ salivary cortisone was significantly higher (AUC difference: 0.83nmol/L.h; p<0.001) in AI/ACS compared to control groups (Figure 3), whereas $AUC_{20:00 - 23:00}$ salivary cortisone in each control group was similar (AUC difference 0.4nmol/L.h; p = 0.4). The correlation between serum cortisol and salivary cortisone was strong (Pearson's r = 0.94). Results for salivary cortisol were less robust both in the correlation between serum cortisol and salivary cortisol (Pearson's r = 0.78) and when comparing AI/ACS with control groups ($AUC_{20:00-23:00}$ difference: 0.67nmol/L.h; p<0.03) (Supplement Figure 2). Salivary cortisol levels were similar between control groups ($AUC_{20:00-23:00}$ difference: 0.28nmol/L.h; p=0.35) (Supplementary Figure 2). Because night-time values for salivary assessments were missing, the fixed-effects components (slope and intercept) of previously described mixed-effects models (18) relating (log) serum cortisol to (log) salivary cortisol or cortisone were used to infer salivary values from measured serum levels through back-calculation (inversion). From this 4-hourly AUCs could be estimated across the full 24 hours. There was no significant difference in 4-hourly AUC salivary cortisone and salivary cortisol between control groups throughout the entire 24-hour period. Calculated salivary cortisone AUC was, however, significantly higher in AI/ACS compared to both control groups between 20:00h and 0:00h (AUC difference 1.1nmol/L.h; p<0.001) and between 22:00h and 02:00h (AUC difference: 0.96nmol/L.h; p<0.001).

Salivary cortisone rhythm post intervention

Post administration of metyrapone 500mg at 18:00h and 250mg at 22:00h $AUC_{20:00 - 23:00}$ salivary cortisone levels in patients with AI/ACS decreased to levels similar to the control groups (AUC difference: -0.12nmol/L.h; p = 0.71) (Figure 3). Similarly between 20:00h and 0:00h (AUC difference -0.1nmol/L.h; p = 0.82) and between 22:00h and 02:00h (AUC difference: 0.10nmol/L.h; p = 0.79) AUC salivary cortisone normalised, such that salivary cortisone rhythm was reset to normal physiological levels.

Il-6 levels pre and post intervention

At baseline serum IL-6 levels were higher in patients with AI/ACS compared to both patients with AI/NoACS and subjects in Group HC at all log converted 4-hourly AUCs between 22:00h and 14:00h (AUC difference 0.42pg/mL.h; p = 0.01). There was no difference in serum IL-6 at any time point between patients with AI/NoACS and HC (p = 0.77). After administration of metyrapone 500mg at 18:00h and 250mg at 22:00h serum IL-6 levels in patients with AI/ACS normalised to levels similar to the other two control groups over all time points over 24 hours (p = 0.08) (Figure 4).

Safety profile

For the AI/ACS group who had intervention 6 adverse events (AEs) were reported by 4 subjects. These included 4 mild headaches (one possibly related), one episode of hypertension and an episode of mild dizziness with a serum cortisol level of 22 nmol/L at 23:00h (levels in normal individuals at this time 5 – 68 nmol/L (18).

Discussion

We have shown that patients with AI/ACS have an abnormal cortisol rhythm with excess evening/nocturnal cortisol exposure and higher IL-6 levels. To our knowledge this is the first time where patients with AI have been investigated by detailed analysis of the serum 24-hour cortisol rhythm. Conversely, an ONDST serum cortisol of 31nmol/L or less in patients with AI is associated with as normal a physiological cortisol rhythm as healthy controls. Groups were carefully matched for age, sex and BMI, so minimising the potential impact that these parameters might have on our observations. Administration of metyrapone specifically in the evening allowed the cortisol rhythm to be 'reset', with an immediate improvement in IL-6 levels; a novel mechanism of action in AI/ACS.

Circadian misalignment, as seen in shift workers, is associated with higher cortisol levels in the evening and higher 24-hour IL-6 levels (22) (23). Moreover, disturbances in the quiescent phase of cortisol secretion in the evening has been associated with impaired glucose tolerance (24), and predicts future new onset Type 2 diabetes (25). In overt Cushing's syndrome circulating IL-6 levels are high, with associated endothelial dysfunction and increased cardiovascular risk (14, 15). Increased circulating IL-6 may cause direct endothelial damage by disturbance of immune and inflammatory processes or through mechanisms mediated by insulin resistance (26), and also impair endothelium-dependent vasodilatation independent of insulin sensitivity (16). Furthermore, higher tertile serum IL-6 is an independent predictor of sudden death in asymptomatic European men (27). In our patients with AI/ACS serum IL-6 levels were high at baseline, but were reduced immediately after 're-setting' of the cortisol rhythm, strongly suggesting that this intervention was causal for the improvement.

Our data show that nocturnal cortisol exposure can be lowered, whilst leaving cortisol levels unaltered throughout the rest of the day. This approach is based on the advantages of using a rapidly acting 11-beta hydroxylase inhibitor with a short duration of action (28) so that the reduction in cortisol could be fine-tuned. This represents an entirely novel paradigm of intervention for these patients for whom debate exists over the best strategy of care (5). Currently, guidelines recommend either observation with treatment to co-morbidities potentially related to cortisol, or adrenal surgery. The problem with the latter is that patients may be referred for surgery inappropriately due to misdiagnosis as there is a high risk for false positivity of diagnostic tests. After metyrapone administration cortisol levels were decreased to the range seen for normal individuals and no adrenal insufficiency event was reported, and, importantly, waking cortisol values were unaffected. Such treatment may allow identification of whether co-morbidities observed in an individual patient improve and so be a means to stratify patients to adrenal surgery or be treated medically in the longer term – personalised precision medicine. A randomised, controlled, prospective study in a

larger sample of AI/ACS patients is needed to assess these notions and to explore the impact of metyrapone treatment on clinical outcomes.

Although an ONDST cut-off of serum cortisol of 50nmol/l is recommended by both the Endocrine Society and European Society of Endocrinology to identify hypercortisolaemia in AI (5, 12) other data suggest that a lower level of serum cortisol may be associated with true normality. Patients with apparently 'non-functioning' AI, with an ONDST serum cortisol level of >30nmol/L, have increased atherosclerosis risk in the absence of conventional cardiovascular risk factors(17). Moreover the prevalence of Type 2 diabetes in a population with no AI is around 14%, similar to patients with AI and an ONDST serum cortisol around 30nmol/L, but is significantly higher in patients with AI and ONDST serum cortisol levels above this value (11). In our study our two control groups showed ONDST serum cortisol of 31nmol/L or less, supporting this cut-point as being associated with a normal cortisol rhythm. The cut-offs used in this study, that is 60nmol/L to 80nmol/L with ACTH <2.2 pmol/L or >80nmol/L, are based on previous data showing increased risk of cardiovascular events and adverse metabolic outcomes with higher post-dexamethasone cortisol levels (8). Furthermore, higher cut-offs than 50nmol/L increases specificity especially in the presence of a suppressed plasma ACTH.

Midnight salivary cortisol is an established means to screen for Cushing's syndrome (12, 29). Despite this, studies in patients with AI have shown that late-night salivary cortisol levels have poor sensitivity for the diagnosis of subclinical hypercortisolism (30, 31). Late-night salivary cortisone may be a better marker for subclinical hypercortisolism in patients with AI, although this needs testing in large cohorts. Additionally, our data show that salivary cortisone may have utility for monitoring of medical intervention with the advantage that it may be used in the community setting without the need for hospital attendance.

The study has limitations: it is small, patients were not dosed over repeated days, and were only studied over 24 hours and therefore no clinical end-points were assessed. IL-6 is known to have

interindividual and intraindividual variability and post-interventional changes could be related to this variability (32). Conversely, the study has been carried out in a stable environment and cortisol and cytokine rhythms were assessed in fine detail. It is the first time that the cortisol rhythm has been systematically studied in this patient group, and control groups were carefully matched.

In summary, we have identified that patients with AI/ACS have an abnormal cortisol rhythm with elevated nocturnal cortisol exposure. This can be 'reset' to normal by using a short acting 11β-hydroxylase inhibitor, metyrapone, in the evening resulting in subsequent decrease in IL-6 levels. This has the potential for an entirely novel approach to intervention in these commonly encountered patients.

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Contributions

MD did the literature search and contributed to data collection, data analysis, data interpretation, and writing of the report. RFH contributed to statistical analysis and data interpretation. RC contributed to study design, data analysis and writing of the report. CG was the research co-ordinator and contributed to writing the report. JLA contributed to study design, data analysis and writing of the report. JNP contributed to study design, was responsible for study oversight and

contributed to data analysis, data interpretation and writing of the report. All authors contributed to the research project and approved its submission.

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Declaration of interests

RC and JLA were HRA PHARMA employees at the time of study conduct and analysis. CG is an HRA PHARMA employee. JNP has received research funding and honoraria from HRA Pharma.

Legends

Figure 1: Adaptive Study Design Figure shows study design highlighting two study phases: baseline analysis (phase 1) and intervention study (phase 2). Interim analysis between phases were organised for research team to analyse data and assess what dose and at what time metyrapone should be administered. At interim analysis 1 all baseline data were analysed and based on the results the decision was taken to administer 500mg of metyrapone at 18:00h. At interim analysis 2 all data from phase 2 were analysed and based on the results the decision was taken to administer 500mg of metyrapone at 18:00h and 250mg at 22:00h.

AI/ACS: Adrenal Incidentalomas with Autonomous Cortisol Secretion; AI/NoACS: Adrenal Incidentalomas with No Autonomous Cortisol Secretion

Figure 2: Serum Cortisol Rhythms

A) Baseline: Concentration-time profiles (geometric mean±SEM) of cortisol rhythm in patients with adrenal incidentalomas and autonomous cortisol secretion (AI/ACS), patients with adrenal incidentalomas and no ACS (AI/NoACS) and Healthy Controls. Higher night-time cortisol exposure between 18:00h and 02:00h is evident in Group AI/ACS. To convert nmol/L to ug/dL divide by 27.59.

B) Reset rhythm after metyrapone: Concentration time profiles (geometric mean±SEM) show that by administering metyrapone 500mg at 18:00h and 250mg at 22:00h one is able to restore the cortisol rhythm to approximate normal physiological concentrations comparable to patients with AI/No ACS and HC. After intervention all 24-hour AUCs of all three concentration time profiles in the three groups of subjects were similar (P=0.29). Log-transformed AUC between 18:00h and 22:00h (P=0.85) and between 22:00h and 02:00h (P=0.76) normalised to physiological levels. To convert nmol/L to ug/dL divide by 27.59.

Figure 3: Concentration time profile for salivary cortisone in the evening (geometric mean±SEM) Patients with AI/ACS have significantly higher salivary cortisone levels than subjects with no ACS (*P*<0.001). Levels are restored to normality after administration of metyrapone 500mg at 18:00h and 250mg at 22:00h. The measurement of salivary cortisone could hence be considered an alternative means to calculate changes in serum cortisol rhythm

Figure 4: Concentration time profiles of serum IL-6 levels pre and post administration of metyrapone in patients with AI/ACS (geometric mean±SEM) Figure indicates higher IL-6 levels in patients with AI/ACS when compared with patients with no ACS (*P*=0.01). Concentrations decrease to normal levels, similar to patients with no ACS after the administration of metyrapone (*P*=0.08).

Supplementary Figure 1 Study Design Figure highlights the number of patients considered for eligibility, the numbers screened and finally recruited to the study. The design includes the number of patients with drawn or excluded and the reasons for this.

Supplementary Figure 2 Baseline Night time Salivary Cortisol (geometric mean ± SEM) Figure shows salivary cortisol levels between 20:00h and 23:00h. When comparing salivary cortisol levels in AI/ACS to control groups (P<0.03) the difference was less evident when compared to salivary cortisone (P<0.001).

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Table

Table 1	Age (yrs)	Weight (kg)	BMI (kg/m²)	Sex	Tumor size (cm)	Dex cortisol (nmol/L)	ACTH (pmol/L)	DHEA (nmol/L)	HT	DM	Statins
A1	60	103.2	43	F	3.5	95	2.0	2.4	Y	Y	N
A2	67	57	24	M	2.7	59	1.6	3.3	N	N	N
A3	61	73.3	29.4	F	2.6	93	1.1	1.6	N	N	N
A4	70	66.3	25	F	1.5	101	3.1	7.0	N	N	N
A5	73	104	32.5	M	3.5	101	6.3	<1.0	Y	N	Y
A6	59	95.3	31.5	M	2.3	127	1.3	2.4	Y	N	Y
B7	62	80.3	33.4	F	2.8	29	2.9	8.0	N	N	Y
B8	62	62.9	25.2	F	0.7	29	1.1	12.0	N	N	N
B9	63	104.9	29.9	M	1.3	22	2.6	5.0	Y	N	Y
B10	65	93.9	31.4	M	2.0	31	4.0	2.6	N	Y	N
B11	65	85.6	36.6	F	2.0	24	4.6	3.3	Y	N	Y
B12	71	57.2	22.9	F	1.8	28	5.1	5.1	N	N	Y
C13	64	109	43.7	F		22	8.4	7.4	N	N	N
C14	72	61.9	24.8	F		29	1.8	18.8	N	N	N
C15	64	113.2	35.7	M		31	4.4	7.1	N	N	N
C16	60	73.1	24.7	M		22	5.3	35.1	Y	N	N
C17	59	58.6	21.5	F		30	2.6	10.9	N	N	N
C18	68	72.5	22.6	M		30	3.1	7.4	Y	N	N

Figure 1

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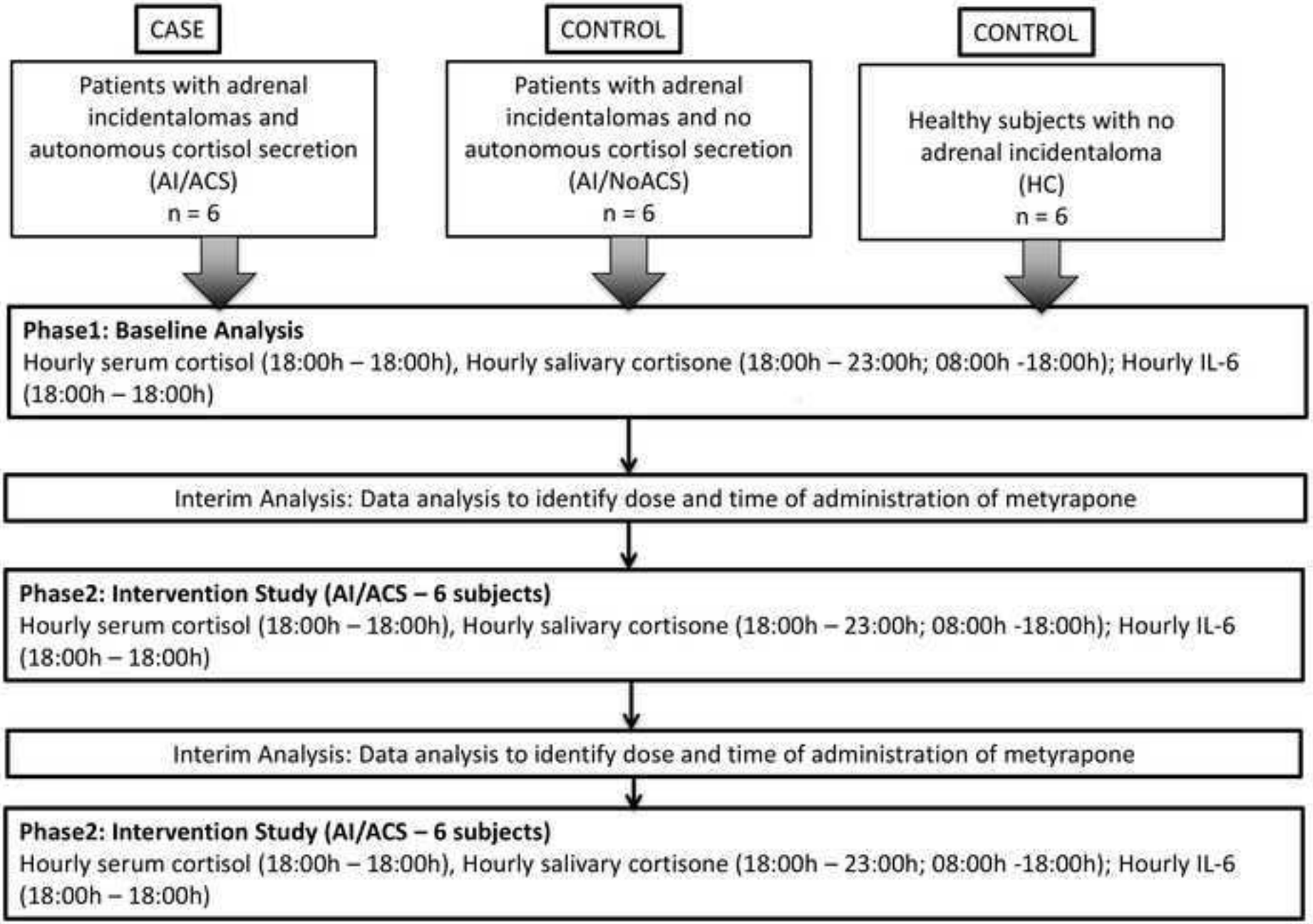

Figure 2

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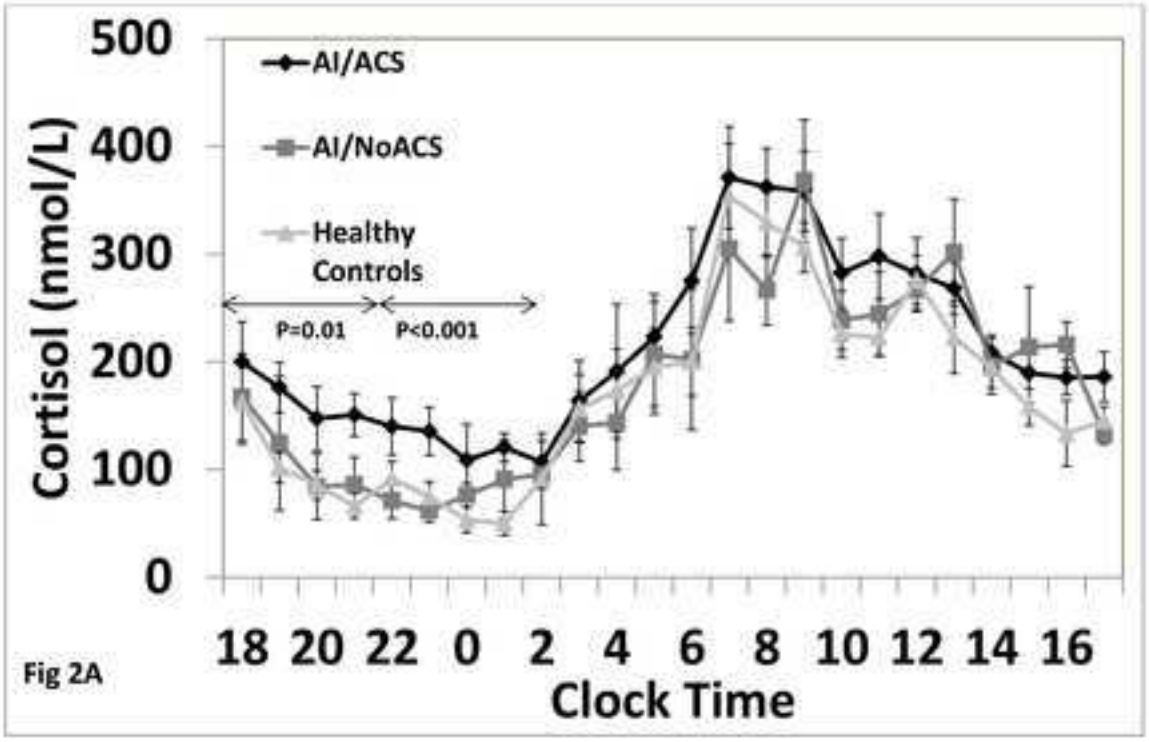

Fig 2A

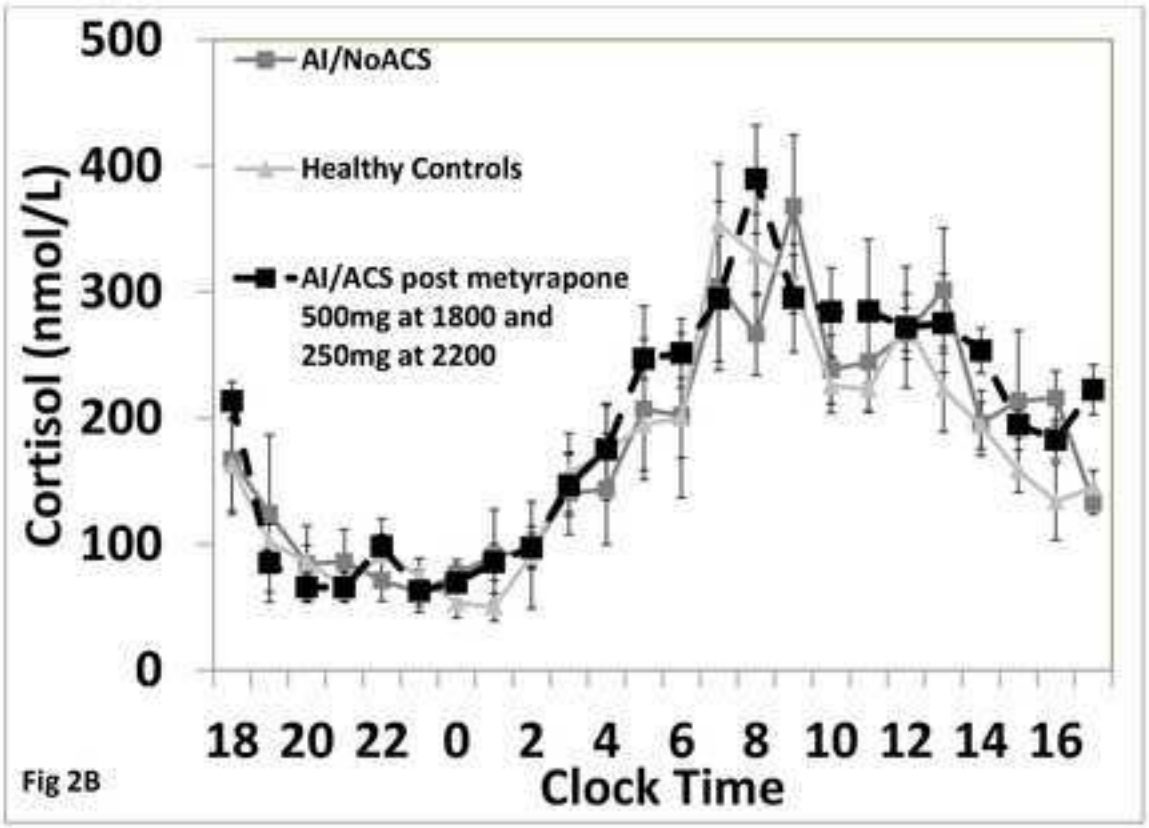

Fig 2B

Figure 3

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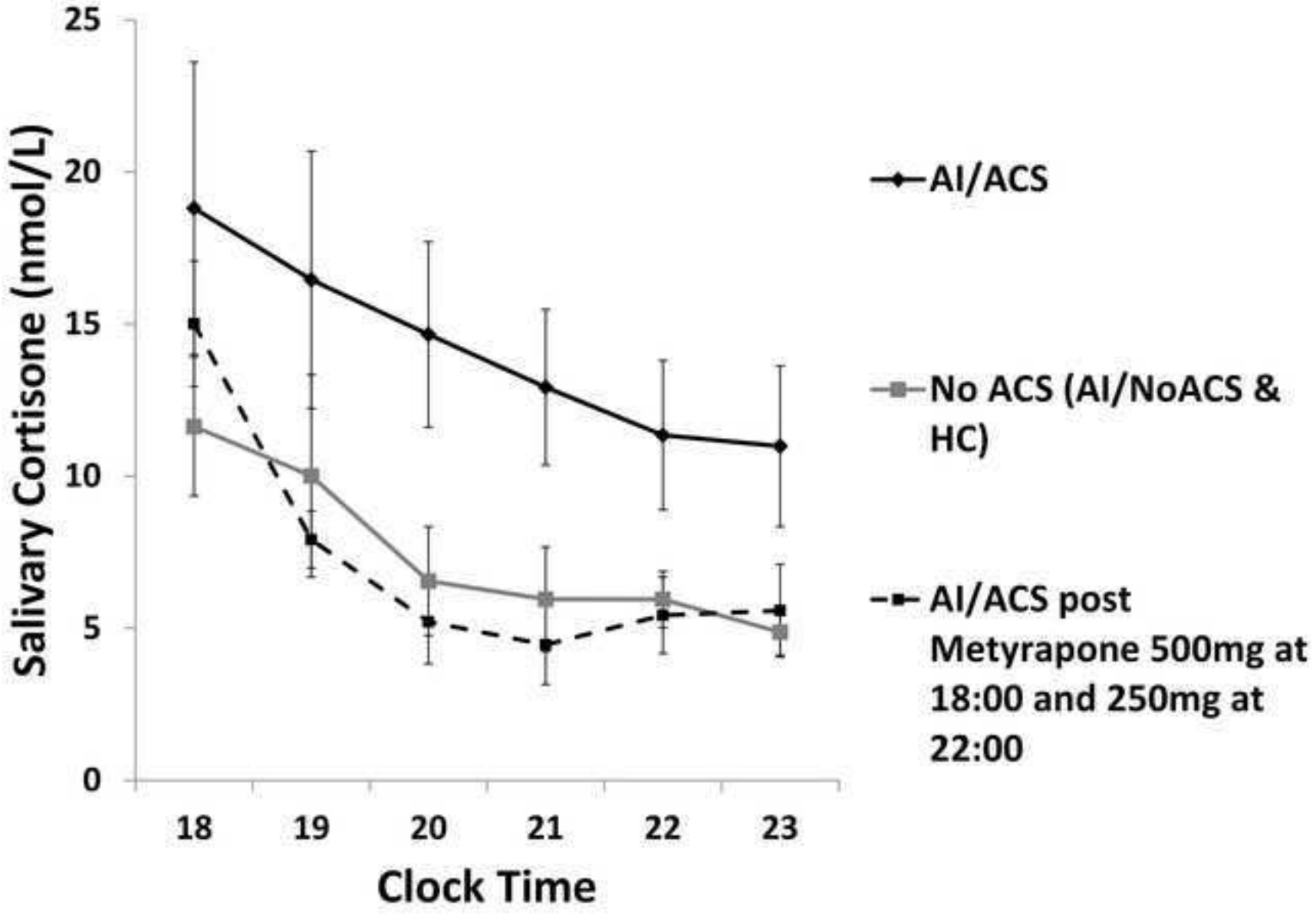

Figure 4

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