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Salivary cortisone to estimate cortisol exposure and sampling frequency required based on serum cortisol measurements

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Salivary cortisone to estimate cortisol exposure and sampling frequency required based on serum cortisol measurements

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Abstract

Context: Population studies frequently measure cortisol as a marker of stress and excess cortisol is associated with increased mortality. Cortisol has a circadian rhythm and frequent blood sampling is impractical to assess exposure. We investigated measuring salivary cortisone and examined sampling frequency required to determine cortisol exposure.

Methods: Serum and saliva with cortisol and cortisone measured by LC-MS/MS in independent cohorts. The relationship between serum cortisol and salivary cortisone was analysed in cohort 1 using a linear mixed effects model and resulting fixed effects component was applied to cohort 2. Saliva cannot easily be collected when sleeping so we determined minimum sampling required to estimate cortisol exposure (eAUC) using 24-hour cortisol profiles (AUC_{24}) and calculated the relative error (RE - a measure similar to the coefficient of variation) for the eAUC.

Results: >90% of variability in salivary cortisone could be accounted for by change in serum cortisol. A single serum cortisol measurement was a poor estimate of AUC_{24} especially in the morning or last thing at night (RE > 68%), however 3 equally spaced samples gave a median RE of 0% (Interquartile range (IQR) between -15.6% and 15.1%). In patients with adrenal incidentalomas the eAUC based on 3 serum cortisol samples showed a difference between those with autonomous cortisol secretion and those without ($p=0.03$).

Interpretation: Accepting that most people sleep 7-8 hours, using approximately 8-hourly salivary cortisone measurements provides a non-invasive method of estimating 24-hour cortisol exposure for population studies.

Introduction

Measuring cortisol exposure is important in defining health. Even a subtle increase in cortisol exposure may affect health outcomes and increased cardiovascular risk and mortality are reported in shift workers and in patients with sleep apnoea and functioning adrenal incidentalomas (1-5). Cortisol deficiency, irrespective of treatment with glucocorticoids, is also associated with elevated mortality rates and a poor quality of life (6,7). In health, serum cortisol demonstrates a distinct circadian rhythm rising from between 0200-0400h to peak shortly after waking and declining over the day to low levels in the evening with a nadir around 2400h (8). Results from a large number of studies from the 1960s to today and using different assays are very consistent regarding this 24-hour rhythm (9). The circadian rhythm of cortisol is altered in shift workers in relation to changes in the sleep-wake cycle and this results in increased cortisol exposure as judged by the 24-hour area under the curve (AUC) of cortisol (10). The same is true for patients with functioning adrenal incidentalomas who have high nocturnal cortisol exposure (11).

The cortisol circadian rhythm has a period of ~24 hours and can be described mathematically by a Fourier Series (cosinor model) (12). Mathematical principles teach us that, in the absence of measurement inaccuracies and other disturbances, the mesor (mean) can be estimated, precisely, by taking the mean of any number of equi-spaced samples exceeding the total number of harmonically related sinusoidal components (harmonics). Because the mesor is proportional to the AUC of a periodic function (AUC = mesor x period) this provides a means of estimating AUC. However, the cortisol circadian rhythm within individuals has biological variability and absolute cortisol levels may be determined by other factors such as genetic sensitivity to glucocorticoids, cortisol production rates and variations in clearance (13-15), but

overall the circadian rhythm of cortisol is similar between populations in different studies (9). Our earlier work suggests that the cortisol rhythm is well modelled by a two-harmonic series (the mesor plus two harmonically-related sinusoidal components) suggesting, therefore, that any three, or more, equi-spaced samples would lead to a reliable estimate of the mesor, hence AUC (16). Given the likely presence of random variation, taking a much larger number of equi-spaced samples would be expected to lead to improved estimates by reducing statistical variability, however, the need to minimise the number of samples in clinical trials militates against this.

Cortisol exposure can be estimated by measuring serum, salivary, interstitial and urine cortisol and each method has its advantages and disadvantages. The measurement of serum cortisol requires venepuncture and the stress of venepuncture may itself raise cortisol levels. Urine requires 24 hour collection, which is often incomplete and in all studies shows reduced sensitivity and specificity for diagnosing cortisol excess compared to measurement of serum samples (17). Interstitial measurements require a complex custom sampling apparatus that is not suitable to study large numbers of subjects. Salivary measurement has the advantage of being non-invasive, can be collected with little stress at home or work and samples are very stable, however sampling cannot be easily done during sleep. Salivary cortisone is emerging as an improved measure of serum cortisol than salivary cortisol, because it is derived from serum free cortisol which is rapidly converted to cortisone in the salivary gland. Salivary cortisone is measurable at low levels of serum cortisol and is not affected by administration of oral hydrocortisone (16,18,19).

Many studies have used single measurements of serum or salivary cortisol to make conclusions about cortisol exposure especially in the field of psychology (20,21), however, in view of the

circadian rhythm of cortisol, these studies are likely to be inaccurate and there is a need for a more accurate estimate of cortisol exposure. We have previously shown that 94% of the variation in salivary cortisone is predicted by changes in serum cortisol (16). We have now tested this relationship between salivary cortisone and serum cortisol in another population of healthy individuals and in a patient population with adrenal incidentalomas some of whom had autonomous cortisol secretion. We have then looked at the frequency of sampling required to estimate the AUC of cortisol over 24 hours using serum cortisol and salivary cortisone.

Methods

Healthy Volunteer and Patient Cohorts: Cortisol data from three previously published cohorts of healthy subjects and patients was used for analysis. Cohorts 1 & 2 had measurements of both serum cortisol and salivary cortisone and were used to examine the relationship between serum cortisol and salivary cortisone and all 3 cohorts had hourly measurement of serum cortisol and were used for analysis of sampling frequency. Meals were not standardised across studies and none of the women were on oestrogen containing therapy:

- Cohort 1: Fourteen healthy male volunteers with a median (interquartile range (IQR)) age of 28 (25 – 36) years, weight 83 (75 – 90) kg and BMI 25.3 (23.1 – 26.3) kg/m^2 who had 24-hour, hourly sampling for serum cortisol and salivary cortisone from 0700-2200h measured by LC-MS/MS (16).
- Cohort 2: Eight patients with adrenal incidentalomas and autonomous cortisol secretion (overnight dexamethasone suppression test serum cortisol >80nmol/L, or 60-80nmol/L with an ACTH <2.2pmol/L (10pg/mL), and no features of clinical Cushing's) and two matched groups (age-, sex- and BMI-matched): 6 patients with adrenal incidentalomas

and no excess cortisol secretion and 6 healthy volunteers. Median (IQR) age of 63 (61 – 67) years, weight 73 (63 – 97) kg and BMI 28 (24 – 33) kg/m^2 who had 24-hour, hourly sampling of serum cortisol and hourly salivary cortisol/cortisone 0600h to 2300h measured by LC-MS/MS (11).

- Cohort 3: 28 healthy (9 female) volunteers mean (range) age 28 (18–56) years who had undergone 24-hour, hourly serum cortisol profiling measured by LC-MS/MS (22).

Assays: LC-MS/MS analysis for serum and salivary cortisone was performed using a Waters Xevo TQ-MS^{TM} mass spectrometer and a Waters Acquity^{TM} LC system with an electrospray source operated in positive ionisation mode (23). The lower limit of quantitation (LLOQ) for serum cortisol was 12.5nmol/L. The inter-assay imprecision was 8, 7 and 6% at concentrations of 80, 480 and 842nmol/L respectively. Salivary cortisone was measured with a modified LC-MS/MS assay with lower limits of detection 0.50 nmol/L, intrassay CVs <7.9%; and interassay <10.3% at 3.6–96 nmol/L of salivary cortisone (24).

Statistical Analysis: All statistical analyses were performed using Matlab^{TM} and Microsoft Excel 2010. In cohort 1, linear mixed effects models were used for both cosinor and regression analysis to account for intra- and inter-subject variability. Model selection was by likelihood-ratio test between models and statistically significant but more complex models with only marginal improvement in either the Akaike or Bayesian Information Criteria were rejected in favour of simplicity. The selected mixed effects model was found to be superior to its fixed effects equivalent ($P<=0.001$). For use in cohort 2, the random effects component of the mixed effects model was no longer applicable and so only the fixed effects element was retained.

AUC estimation was conducted as follows. AUC_{24} was computed by the trapezium rule. One sample estimated AUC (eAUC): was computed as 24 times the sampled value. Two sample

eAUC: the earliest start-time was selected and the mean of the corresponding sample and the sample 12 hours later was computed and multiplied by 24. The start point was advanced by one hour and repeated until the sample was exhausted. Three sample eAUC: As above with samples at baseline, 8 & 16 hours and likewise for four samples.

To account for inter-subject variability we derived the relative error (RE), a measure similar to the coefficient of variation. For each subject we computed the difference between the actual AUC (AUC_{24}) and the under-sampled estimates, (eAUC) and divided by the AUC_{24}, thus removing the inter-subject effect.

The sensitivity analysis explored the loss of accuracy (deviation from eAUC) that occurs when samples are not taken at their prescribed times. This was done by taking all possible patterns of sampling one hour too early or too late and computing the relative deviation from the "on-time" estimate.

A two-sample t-test with unequal variances was used to examine differences between patients with adrenal incidentalomas with and without subclinical hypercortisolism.

Ethics: All subjects and patients gave full informed consent: cohort 1, the study received approval from the South East Wales Research Ethics Committee; cohort 2, the study received approval from East Leeds National Research Ethics Service Committee, cohort 3, the study was approved by the South Manchester Local Research Ethics Committee.

Results

Relationship between salivary cortisone and serum cortisol (figure 1): Application of the fixed effects model: $\log_{10}$ serum F = 1.24+0.89 $\log_{10}$ salE describing the relationship between serum cortisol and salivary cortisone in cohort 1 was applied to cohort 2 which included patients with adrenal incidentalomas with autonomous cortisol secretion as well as matched controls. The fixed effects model from cohort 1 gave similar results in cohort 2: model predictions of serum cortisol from salivary cortisone gave correlation coefficients of r=0.93 and 0.91 $p<0.001$, for cohorts 1 & 2 respectively.

Frequency of serum cortisol sampling and comparison of eAUC vs AUC_{24} (figure 2 & table 1): A single sample used to calculate the eAUC was a very poor predictor of the AUC_{24}, especially in the morning and last thing at night. The median RE were greatest between 0700-0900h and 2300-0100h, being 104% to -68%, and the smallest values were between 0400-0500h and 1400-1600h, being -42% to 30%. The RE falls as 2, 3 and 4 equi-spaced samples are used to calculate the eAUC with the IQR for the RE with 3 equi-spaced samples being -15.6% to 15.1%, and for 4 equi-spaced samples -14.3% to 11.4%. The same pattern was seen when the individual cohorts were analysed (Table1).

Sensitivity analysis on timing of samples: The 8-hourly sampling scheme is relatively insensitive to mistiming of the samples by up to one hour either way for any or all samples. Looking at the variation of the mistimed (± one hour) 3-sample eAUCs against the eAUC on-time across all three cohorts gives a median RE of 0% with IQR between -7.3% and 7.6%.

Comparison of eAUC vs AUC_{24} in patients with adrenal incidentalomas with and without autonomous cortisol secretion (Figure 3): To test whether the eAUC could be used to distinguish different patient populations we examined the AUC24 and eAUC between healthy controls and patients with adrenal incidentaloma and autonomous cortisol secretion and those without autonomous cortisol secretion. There was a difference between AUC_{24} for patients with adrenal incidentalomas and autonomous cortisol secretion and those without ($p<0.02$) and the same pattern was seen for eAUC based on 3 serum cortisol samples ($p=0.03$) and although the eAUC based on 3 salivary cortisone samples didn't reach significance ($p=0.06$) the pattern was the same. The 3 samples used for serum cortisol were 0700, 1500, & 2300h but as there was no salivary sample at 0700h the 3 samples used for salivary cortisone were 0800, 1500 & 2300h. The 2300h salivary cortisone in patients with and without hypercortisolaemia showed that the 2300h salivary cortisone was higher in the patients with subclinical hypercortisolaemia: median (25-75 percentiles) controls 4.5, 4.0-7.9; SCH 9.9, 7.5-16.7 AI 4.4, 3.0-7.4 ANOVA $p=0.03$ with SCH different from AI and controls $p<0.05$. For the healthy men in cohort 1 the eAUC for salivary cortisone median (25-75 percentiles) was 406 (387-470) nmol hours/L similar to that of the patients with adrenal incidentalomas and no autonomous cortisol secretion.

Discussion

We have confirmed that salivary cortisone is a good estimate of serum cortisol in populations of both healthy subjects and patients. Examining the frequency of serum cortisol sampling, we demonstrate that a single cortisol sample is a poor measure of cortisol AUC, especially when taken around the time of waking and going to sleep. However, three equi-spaced 8 hourly serum cortisol samples give an eAUC with an inter-quartile range between -15.6% and 15.1%. of the AUC_{24} and this was relatively insensitive to mistiming by one hour. Taken together these results suggest that three approximately 8 hourly spaced salivary cortisone measurements can give a good estimate of serum cortisol exposure in healthy and patient populations and provides an algorithm for measuring 24 hour cortisol exposure without interrupting sleep and that is independent of the time of starting sampling.

It is evident from our data that the single measurement of cortisol when taken in the morning or last thing at night has a poor correlation with overall 24-hour cortisol AUC. This is in accordance with the problem of AUC estimation from a small number of samples in data that has a periodic component. Estimates can only be unbiased if the number of samples exceeds the number of significant harmonic components needed to represent the curve, two in the case of cortisol. A single sample will always be biased unless its timing matches the point at which the curve crosses the mesor. From our data, the best times for a single measurement in relation to overall cortisol exposure is when the RE is lowest between either 1400–1600h in the afternoon or 0400–0500h in the morning corresponding to when the cortisol rhythm crosses the mesor as predicted by theory. Timing of a single sample is tricky in shift workers whereas taking 3 approximately 8 hourly samples allows sampling to start at any time. The cortisol circadian rhythm is described mathematically by a sinusoid with two harmonics and

as such 3 or more equally spaced samples taken over 24 hours should correlate well with the AUC_{24}. This is what we observed. Increasing the number of samples will reduce variability in the estimates, however, 6 hourly or more frequent sampling is impractical because it would require sampling during sleep. We found that there was little difference in the accuracy of predicting the AUC_{24} between 8 hourly vs 6 hourly sampling, and even when samples were not taken exactly 8 hourly we found there was still a good correlation between the eAUC and AUC_{24}.

We are not proposing the salivary cortisone eAUC as a diagnostic test for Cushing's syndrome and adrenal insufficiency, where we already have specific and sensitive tests and cortisol levels at specific times of the day are more relevant than the 24 hour cortisol exposure. The single measurement of either serum or salivary cortisol as a diagnostic test has been used in many studies to investigate Cushing's syndrome and disease (17,25-27). A single late night cortisol measurement is a sensitive method for diagnosing Cushing's syndrome and has been shown to be elevated in some populations such as those with type 2 diabetes (27), and in our study the single measurement of salivary cortisone at 2300h did differentiate function from non-functioning adrenal incidentalomas. However, cortisol exposure (24h cortisol AUC) varies in both patients with Cushing's syndrome, adrenal insufficiency and there is overlap between patient populations and healthy individuals. A recent study in patients with Cushing's disease showed great variability in late-night salivary cortisol within patients over time (28) and late night salivary cortisol is a poor marker differentiate functioning from non-functioning adrenal incidentalomas (29). We propose that the salivary cortisone eAUC provides an easy to administer more accurate method for comparing cortisol exposure in populations of patients or healthy subjects than single samples or 24 hour serum profiles.

In our small cohort of patients with functioning adrenal incidentalomas, if samples were taken in the morning the excess cortisol secretion would be missed whereas, as shown by our data, a sample taken last thing at night or 3 samples taken approximately 8 hourly demonstrated that adrenal incidentalomas with excess cortisol secretion, as judged by a dexamethasone suppression test, had overall increased cortisol secretion compared to non-functioning adrenal incidentalomas. It is likely that adrenal tumours have more stable cortisol excretion whereas in Cushing's disease there may be variability over time, however 3 samples rather than 1 is likely to better define the variability related to disease. The salivary cortisone eAUC in the healthy men in cohort 1 was similar to that of patients with non-functioning adrenal incidentalomas, however this is not a normal range as a much bigger sample of the population would be required. We know in any population of healthy individuals and patients there is variation in 24-hour cortisol exposure and overlap between patients with excess and deficient cortisol secretion. Therefore, We know that meal times, shift work and stress can influence cortisol exposure so in studies comparing populations it is important to control for these factors.

Salivary cortisol has been used as a measurement of free cortisol since the 1960s (30) and now LC-MS/MS provides a highly specific and sensitive method whereby we can measure cortisol and cortisone simultaneously (31). Free serum cortisol is rapidly converted to cortisone in the salivary gland and salivary cortisone generally shows a better correlation with serum cortisol than salivary cortisol especially at low levels of serum cortisol where salivary cortisol is undetectable (19). We have previously shown that salivary cortisone reflects serum cortisol using a mixed effects model and we have now shown that its fixed effects component demonstrates an almost identical relationship in another healthy volunteer

population as well as in patients being investigated for adrenal incidentalomas, half of whom had functioning adrenal adenomas secreting cortisol. The results confirm that salivary cortisone is a good method for estimating serum cortisol levels and further studies are required to establish its use. Saliva has the advantage of being non-invasive, can be collected in a non-clinical setting and, because steroids are very stable, samples can be posted to the laboratory without any special conditions.

Limitations of our data are the retrospective analysis and that the patient population is relatively small. This is reflected in the fact that the difference in eAUC for salivary cortisone between patients with adrenal incidentalomas with or without excess cortisol secretion didn't reach significance. However, the studies analysed provide comprehensive data of hourly sampling over 24 hours in 3 different subject cohorts and the results are consistent over the different cohorts. Although this analysis is retrospective all the studies were done under carefully monitored controlled conditions. It should be recognised that two AUCs can be the same but the rhythm may be different but it is difficult to define the rhythm from limited sampling and this will generally require more frequent sampling.

This study provides a strong basis for using three approximately 8 hourly spaced salivary cortisone samples when estimating cortisol exposure in healthy and patient populations. This methodology will allow further investigation of the impact of cortisol secretion on health.

Legends

Table 1: Relative Error (RE) for individual cohorts.

Figure 1: Shows the relationship between serum cortisol and salivary cortisone. Cohort 1 was analysed using a linear mixed effects model and the resulting fixed effects component was applied predictively to Cohort 2. The relationship is the same in both cohorts.

Figure 2: Box-plots of Relative Error with IQR and range across all cohorts using 1 to 4 equi-spaced sampling points in estimating AUC. The size of the Relative Error and variation over time decreases with the increasing number of samples measured.

Figure 3. AUC_{24} (a), eAUC for serum cortisol (b) and eAUC salivary cortisone (c) based on 3 approximately equi-spaced samples in patients with Subclinical HyperCortisolism (SCH) and without autonomous cortisol section (AI) and adrenal incidentalomas. Boxes show IQR and dotted lines minimum and max range.

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Table 1: Relative Error (RE) for individual cohorts.

3-equi-spaced samples RE (%)

	median	25%ile	75%ile	IQR
Cohort 1	2.4	-16.5	20.7	37.1
Cohort 2	-1.69	-2.37	1.55	3.92
Cohort 3	-5.78	-11.7	9.39	21.1
All	-0.03	-15.6	15.1	30.6

4-equi-spaced samples RE (%)

	median	25%ile	75%ile	IQR
Cohort 1	2.3	-6.61	6.77	13.4
Cohort 2	-1.53	-7.23	3.37	10.6
Cohort 3	-0.445	-12.1	5.05	17.2
All	-1.11	-14.3	11.4	25.7

Figure 1

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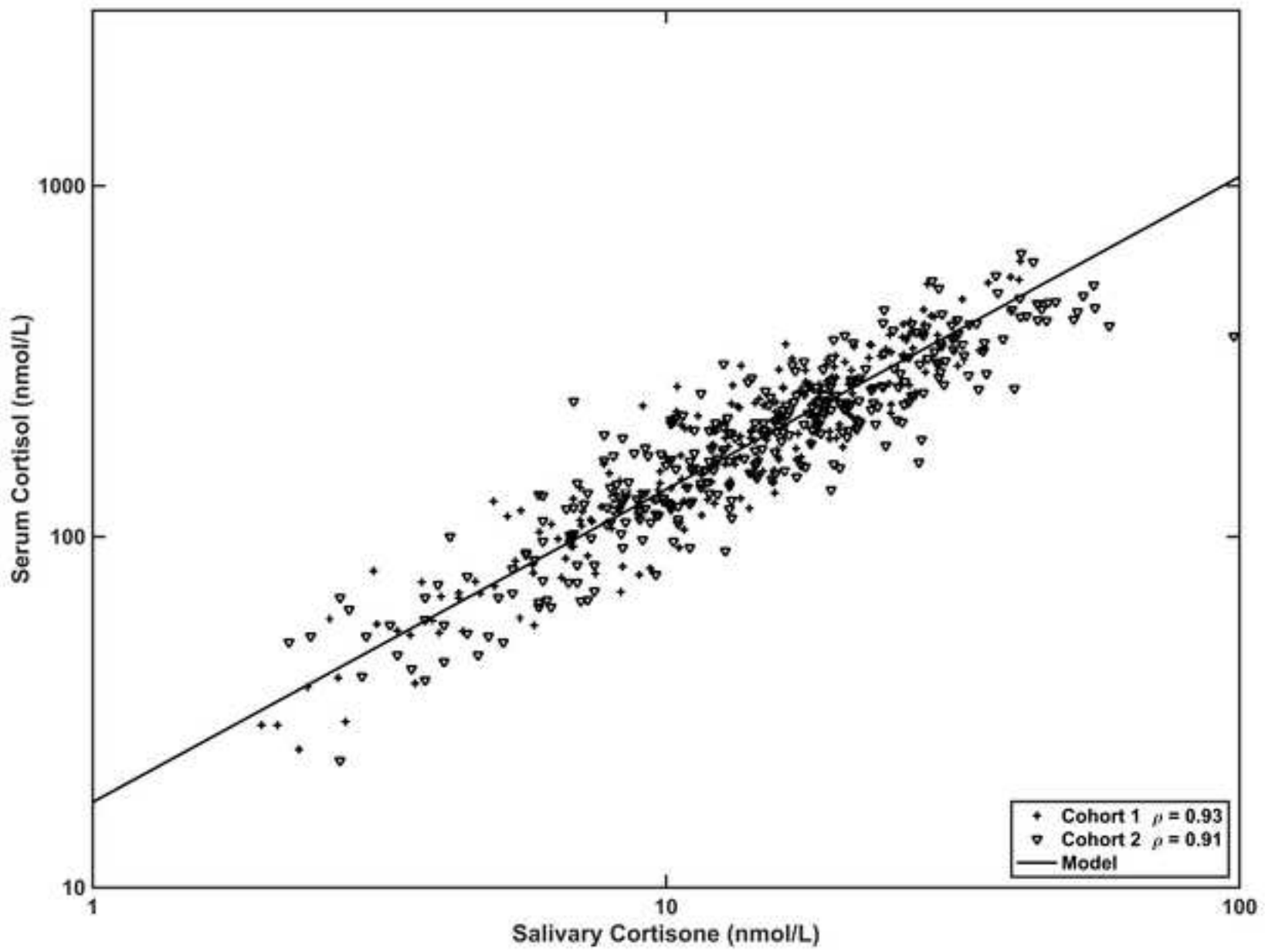

Figure 2

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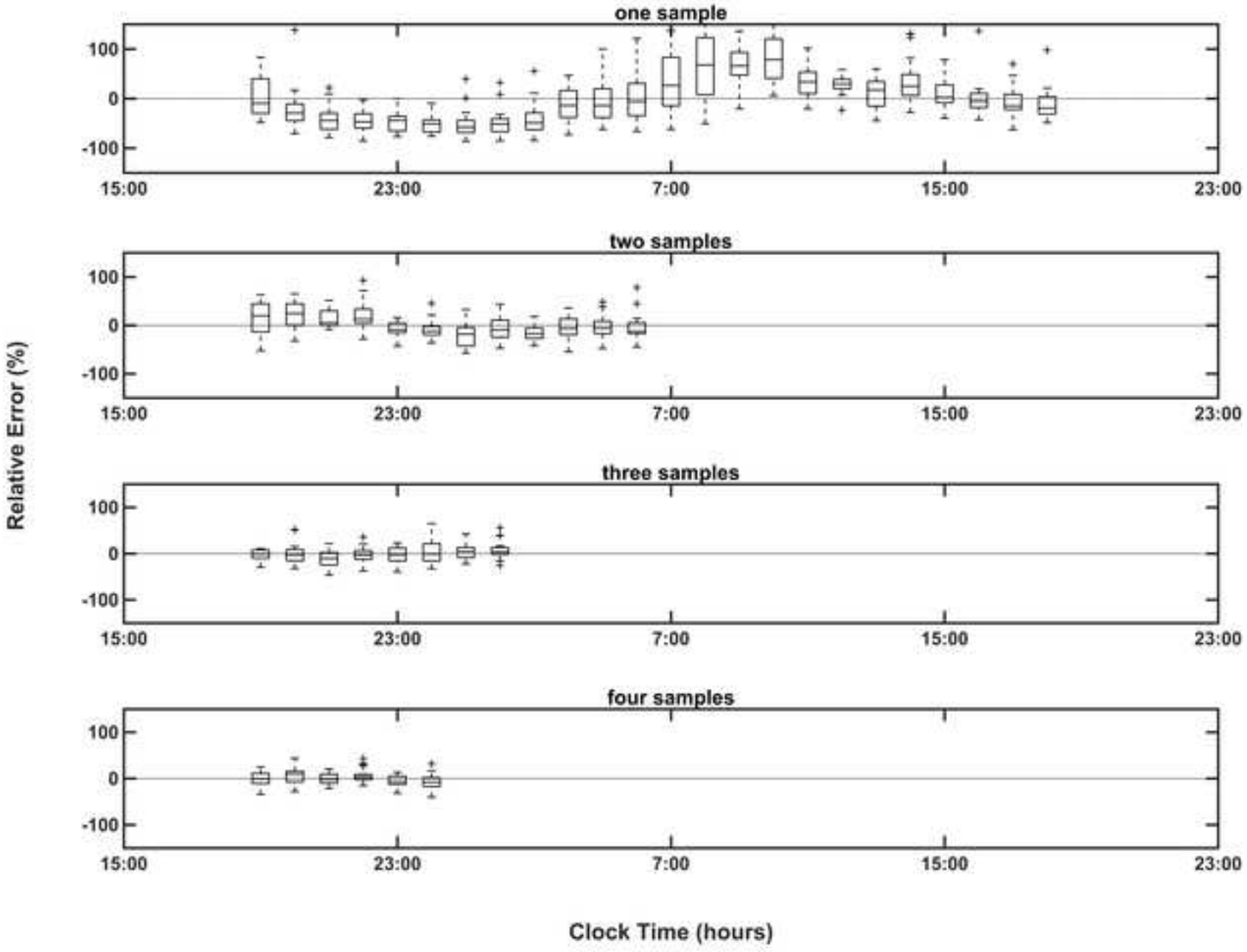

Figure 3

Click here to access/download;Figure;Figure_3.tif