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Diurnal patterns and associations among salivary cortisol, DHEA and alpha-amylase in older adults☆

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Abstract

Background—Cortisol and dehydroepiandrosterone (DHEA) are considered to be valuable markers of the hypothalamus–pituitary–adrenal (HPA) axis, while salivary alpha-amylase (sAA) reflects the autonomic nervous system. Past studies have found certain diurnal patterns among these biomarkers, with some studies reporting results that differ from others. Also, some past studies have found an association among these three biomarkers while other studies have not. This study investigates these patterns and associations in older adults by taking advantage of modern statistical methods for dealing with non-normality, outliers and curvature. Basic characteristics of the data are reported as well, which are relevant to understanding the nature of any patterns and associations.

Methods—Boxplots were used to check on the skewness and presence of outliers, including the impact of using simple transformations for dealing with non-normality. Diurnal patterns were investigated using recent advances aimed at comparing medians. When studying associations, the initial step was to check for curvature using a non-parametric regression estimator. Based on the resulting fit, a robust regression estimator was used that is designed to deal with skewed distributions and outliers.

Results—Boxplots indicated highly skewed distributions with outliers. Simple transformations (such as taking logs) did not deal with this issue in an effective manner. Consequently, diurnal patterns were investigated using medians and found to be consistent with some previous studies but not others. A positive association between awakening cortisol levels and DHEA was found when DHEA is relatively low; otherwise no association was found. The nature of the association

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between cortisol and DHEA was found to change during the course of the day. Upon awakening, cortisol was found to have no association with sAA when DHEA levels are relatively low, but otherwise there is a negative association. DHEA was found to have a positive association with sAA upon awakening. Shortly after awakening and for the remainder of the day, no association was found between DHEA and sAA ignoring cortisol. For DHEA and cortisol (taken as the independent variables) versus sAA (the dependent variable), again an association is found only upon awakening.

Keywords

Cortisol; Dehydroepiandrosterone (DHEA); Salivary alpha-amylase; Well Elderly II study

1. Introduction

The hypothalamus–pituitary–adrenal (HPA) axis and the sympathetic–adrenomedullary system (SAM) are two major biological systems involved in homeostatic and allostatic adaptations to environmental and internal stimuli [37,23,42,12,43]. Dysfunction in the HPA axis is implicated in the development of a variety of sub-clinical and clinical conditions [43,44,3]. Cortisol, the primary hormone product secreted by the HPA axis, is considered to be a biomarker of HPA axis activity (e.g., [22]). Dehydroepiandrosterone (DHEA) is also secreted by the adrenal cortex and plays a pivotal role in the regulation of HPA activity, with effects that are opposite to cortisol at both peripheral and central levels [40,14,17].

Past studies have found that mean cortisol levels exhibit an initial rise after awakening, referred to as the cortisol awakening response (CAR), followed by a decline in cortisol during the remainder of the day. Pruessner et al. [53] were the first to propose that the repeated assessment of salivary cortisol increase after awakening might represent a useful and easy measure index of cortisol regulation. In most studies, an increase in salivary cortisol levels of about 50–75% within 30–45 min after awakening has been found. The CAR is increasingly used in psychoneuroendocrinology as an indicator of HPA axis activity. For reviews of the literature, see Clow et al. [11], Chida and Steptoe [8] and Fries et al. [20]. The CAR is considered to be a marker of the integrity of the HPA axis [29]. Exhibiting an absence or an exacerbation of this increase is associated with several adverse psychological and physiological outcomes (e.g., [51,50]). Both enhanced and reduced CARs are associated with various psychosocial factors [36,8], including depression and anxiety disorders (e.g., [52,64,4,65,66]).

Currently, only a few studies have reported results about the daily fluctuations of DHEA and sAA. Regarding DHEA, a circadian variation (with a trough concentration later in the day) has been reported in adults [72,22] but not in the elderly [13].

The CAR is considered a reliable parameter for detecting participants who are non-adherent to a study protocol [63,61,46]. Consequently, in a study focusing on young adults, Ghiciuc et al. [22] examine diurnal patterns for participants who exhibited cortisol increases by at least 50% after awakening and found that mean DHEA levels decreased significantly 15 min after awakening. A non-significant increase was observed at 30 and 45 min and a significant increase was reported about 13 h after awakening. The rationale for focusing only on

participants with a CAR of 50% was that failure to comply with the strict timing of saliva sampling can influence hormonal measurements and compromise the accuracy and reliability of the results [27,38].

Secretion of sAA has been proposed as an indicator of plasma catecholamine modifications under a variety of conditions [7,67,62,39,70]. Significant diurnal fluctuations in sAA have been reported, with low values reached within 60 min after awakening [45] and much higher values reached later in the day [47,2,71,45,22].

Very little is known about the associations among cortisol, DHEA and sAA, particularly for older adults. The aim of our study is to help fill this gap by reporting results on the associations among cortisol, DHEA and sAA in the well elderly. A unique feature of the present study is the use of modern statistical methods that provide improved techniques aimed at dealing with outliers, non-normality and curvature. Another goal was to provide new information and perspectives regarding the diurnal patterns of cortisol, DHEA and sAA.

2. Material and methods

2.1. Participants and study design

The data stem from the Well Elderly II study [9,34]. The participants were 460 men and women aged 60 to 95 years (mean age 74.9). All participants were residents of, users of, or visitors to the study recruitment sites, demonstrated no overt signs of psychosis or dementia (based on a cursory screening procedure), and were able to complete the study assessment battery (with assistance, if necessary). All prospective participants completed the informed consent process prior to study entry. Participants were recruited from 21 sites in the greater Los Angeles area, including nine senior activity centers, eleven senior housing residences, and one graduated care retirement community. Recruitment strategies included providing sign-up booths, giving presentations at meetings and social events, and distributing flyers and posters. Recruitment was undertaken in two successive cohorts to reduce temporal influences on study outcomes, overcome logistical difficulties, minimize interactions among participants, and allow adjustments in ethnic stratification. Individuals in cohort 1 (n = 205) began participation between November, 2004 and June, 2005, whereas those in cohort 2 (n = 255) began participation between March and August, 2006. Here, the two cohorts are combined in all analyses.

2.2. Assessment

Saliva samples were taken at four times: upon awakening, 30–45 min later, 5 h later, and 5 h later. Samples were assayed for sAA using a commercially available kinetic reaction assay (Salimetrics, State College, PA). The assay employs a chromogenic substrate, 2-chloro-p-nitrophenol, linked to maltotriose. The enzymatic action of sAA on this substrate yields 2-chloro-pnitrophenol, which can be spectrophotometrically measured at 405 nm using a standard laboratory plate reader. The amount of activity present in the sample is directly proportional to the increase (over a 2 min period) in absorbance at 405 nm. Results are computed in units per milliliter of sAA using the formula: [Absorbance difference per

minute × total assay volume (328 ml) × dilution factor (200)] / [millimolar absorptivity of 2-chloro-p-nitrophenol (12.9) × sample volume (.008 ml) × light path (.97)].

Samples were assayed for cortisol using a highly sensitive enzyme immunoassay. The test uses 25 μl/dl, ranges in sensitivity from .007 to 1.2 μl/dl, and has average intra- and inter-assay coefficients of variation of 4.13% and 8.89%, respectively. Samples were assayed for salivary DHEA using a double antibody radioimmunoassay developed at Penn State Behavioral Endocrinology Laboratory (Granger et al., 1999). The test uses 100 μl of saliva, has a minimum detection limit of 4 pg/ml and average intra- and inter-assay coefficients of variation less than 4.05% and 12.57%, respectively.

2.3. Data analysis and statistics

All analyses were performed with the software R [54]. The median levels of the biomarkers were compared using a percentile bootstrap method that deals effectively with tied (duplicated) values (e.g., [68], Section 5.9.11). As will be seen, the data in the present study are skewed with outliers suggesting that conventional methods for comparing means can have relatively low power and poor control over the Type I error probability (e.g., [58,41,30,32,68,26]). Indeed, even a single outlier might result in poor power. Nonparametric (rank-based) methods are sometimes suggested for comparing medians. There are exceptions, but under general conditions, nonparametric methods do not compare medians (e.g., [21,31,6]). The simple strategy of transforming the data is relatively ineffective by modern standards in terms of dealing with outliers and skewed distributions. For example, taking logs, typically outliers remain and distributions are still skewed, which proved to be the case for the data at hand.

When using least squares regression, again outliers can wreak havoc on power and they can result in a highly misleading summary of the association among the bulk of the points. Here, outliers among the dependent variable were addressed using the regression estimator derived by Theil [60] and Sen [59], which is designed to estimate the median of a dependent variable rather than the mean. As is the case when dealing with means, simply discarding outliers among the dependent variable and applying least squares regression to the remaining data generally yields poor control over the probability of a Type I error. As for the independent variable, theory allows one to remove outliers. This was done here using an outlier detection method that has been studied extensively in the statistics literature (e.g., [56,69]). These books also explain why outlier detection techniques, based on the mean and variance, are highly unsatisfactory. Multivariate outliers were detected using the method in Wilcox ([69], Section 6.4.7).

The usual linear model assumes the regression line is straight, but modern methods make it clear that often this assumption is unsatisfactory. Moreover, the more obvious parametric methods for dealing with curvature can be unsatisfactory relative to more recently derived techniques. For a summary of the many details and various methods for dealing with curvature, often called smoothers or nonparametric regression estimators, see for example [28,15,16,19,24]. The method used here is generally known as LOESS and was derived by Cleveland and Devlin [10]; it was applied with the R function lplot [69], which returns a

correlation coefficient that reduces to Pearson's correlation when using least squares regression.

The familywise error rate (the probability of one or more Type I errors) was set at .05 and controlled using the method derived by Rom [55], which improves on the Bonferroni method.

3. Results

Evidence of skewness and outliers is provided by the boxplots in Fig. 1. Shown are the measures upon awakening, 30–60 min later, approximately 5 h later and another 5 h later. About 10% of the values are flagged as outliers. (Taking logs, these proportions drop slightly.) Fig. 2 shows the medians (with the bars indicating a distribution-free .95 confidence interval). For cortisol, all pairwise comparisons of the medians are significantly different, indicating that typical cortisol measures increase shortly after awakening and then decline during the day, consistent with past studies. For DHEA and sAA all pairwise comparisons are significant except for times 3 and 4. That is, typical DHEA values tend to decline during the early portion of the day, after which no significant change was found. As for sAA, the typical level drops shortly after awakening and then tends to increase during the early portion of the day, but no significant changes were found otherwise.

Fig. 3 shows an estimate of the regression line between awakening cortisol and DHEA ignoring outliers. The strength of the association was estimated to be .32. Note that there appears to be a change in the nature of the association around where cortisol is equal to .4 μg/ml. For cortisol less than .4 μg/ml, a positive association is found, but no association is found when cortisol is greater than .4 μg/ml. Comparing the slopes corresponding to these two situations, a significant result is not obtained ($p = .11$). Splitting the data based on whether cortisol is less than .57 μg/ml, the slopes differ significantly ($p = .003$). As for the other three times, there is no indication of a bend as shown in Fig. 3; all three regression lines appear to be approximately straight with a positive slope that differs significantly from zero. The strength of the association corresponding to these three times were .46, .35 and .43, respectively.

Fig. 4 shows an estimate of the regression surface when predicting sAA upon awakening using both cortisol and DHEA. Fig. 4 indicates that typical sAA values tend to be highest when simultaneously cortisol is low and DHEA is relatively high. Fitting a regression plane, both slopes are positive with the slope for cortisol non-significant ($p = .81$) and the slope for DHEA significant ($p = .01$).However, Fig. 4 suggests that curvature might be an issue with the nature of the association changing as DHEA increases. To check on this possibility, the data were split into two groups according to whether DHEA is less than or greater than 100 pg/ml. For DHEA less than 100 pg/ml, no association is found. But for DHEA greater than 100 pg/ml, the slopes for both cortisol and DHEA are significant ($p = .037$ and .007, respectively). Now the slope associated with cortisol is negative and it differs significantly from the slope when DHEA is less than 100 pg/ml ($p = .027$).

4. Discussion

Despite the degree of skewness and the outliers indicated in Fig. 1, the diurnal patterns in older adults, based on medians, are consistent with the most past studies (e.g., [1,22,33,53,72]). Moreover, we found significant relationships among these salivary biomarkers that in some instances are not described well with the typical linear models.

Regarding the diurnal pattern of sAA, the results reported here are consistent with Nater et al. [45] who reported sharp drops in salivary sAA activity 30 and 60min after waking. This is in contrast to the results reported by Ghiciuc et al. [22] who found that sAA declined 15 min after awakening followed by a non-significant increase 30 and 45 min later. Ghiciuc et al. speculated that this slight discrepancy might be related to different time schedules or perhaps the cortisol awakening response. A partial check on this latter possibility was made in two ways. First, sAA levels were compared again using only participants whose cortisol levels increased shortly after awakening. Again the median sAA levels decreased significantly at time 2 ($p < .001$). This was followed by a significant increase from time 2 to time 3 ($p < .001$). Using means rather than medians yielded the same result. Next, as was done by Ghiciuc et al., the analysis was run based on only those participants whose awakening cortisol increased by 50%. The results were the same as just described. (For time 1 vs. time 2, $p = .033$, and for time 2 vs. time 3, $p < .001$.) Another possible explanation for this discrepancy with the results in Ghiciuc et al. is that they focused on young adults rather than older adults as done here.

Our results are in agreement with other studies that have reported a DHEA concentration trough during the day [1,22,72].

The results on the association between cortisol and DHEA are consistent with Boudarene et al. [5] who found a significant positive association via Spearman's correlation. Here, a positive association was found at each time measures were taken. However, it was found that upon awakening, for higher levels of cortisol, the association weakens and becomes non-significant. This was not found to be the case for the other times.

Some past studies have found no correlation between cortisol and sAA responses to various stress paradigms [7,45,18]. Here an association between cortisol and sAA is found when measures are taken upon awakening and when DHEA levels are taken into account. When DHEA is low, no association between cortisol and sAA was found. At awakening, when DHEA levels tend to be relatively high, cortisol has a negative association with sAA. No association between cortisol and sAA was found at later times, possibly because DHEA levels are lower than they are at awakening.

The measurement of salivary cortisol, sAA and DHEA is becoming more widely accepted for monitoring changes in HPA and SAM activity under stress-related conditions [35,36,48,49,57]. Changes in salivary cortisol, sAA and DHEA levels, as well as their diurnal fluctuations, are thought to have implications for health [1,70,8]. The results reported here support the conclusion reached by other studies that an appropriate time of day for sample collection is important when investigating neuroendocrine changes under different conditions [25,22].

Finally, by taking advantage of modern robust statistical methods aimed at dealing with outliers and curvature, the nature of the association among these three biomarkers was found to be more complex than indicated in past studies. While the goal here was to characterize the typical measures and how they are related, it is prudent to keep in mind that about 10% of the responses are highly unusual compared to the bulk of the participants. Of interest is whether modern methods aimed at dealing with outliers make a practical difference in other settings.

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HIGHLIGHTS

- We studied diurnal patterns and associations among cortisol, DHEA and α-amylase (sAA).
- Diurnal patterns, based on medians, were similar to some past studies but not others.
- The nature of the associations depends on the time of day.
- The association between awakening cortisol and sAA depends on the level of DHEA.
- After awakening, no association between cortisol, DHEA versus sAA is found.

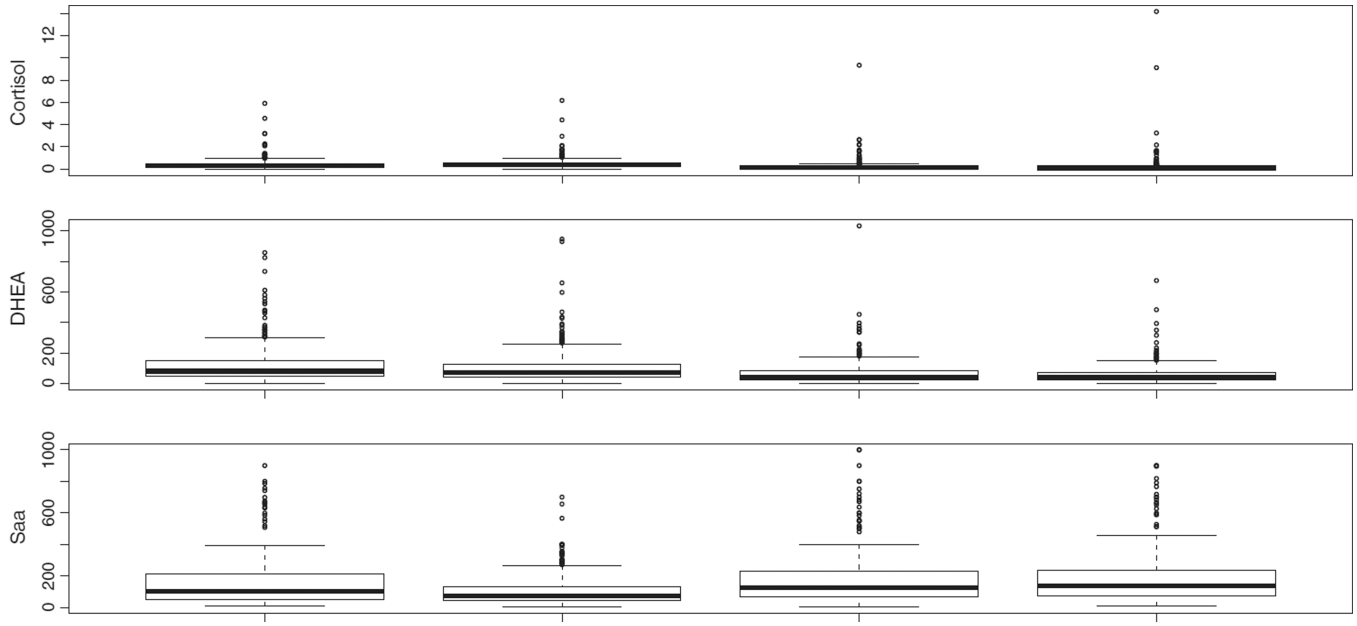

Fig. 1.
Boxplots of the cortisol, DHEA and sAA data.

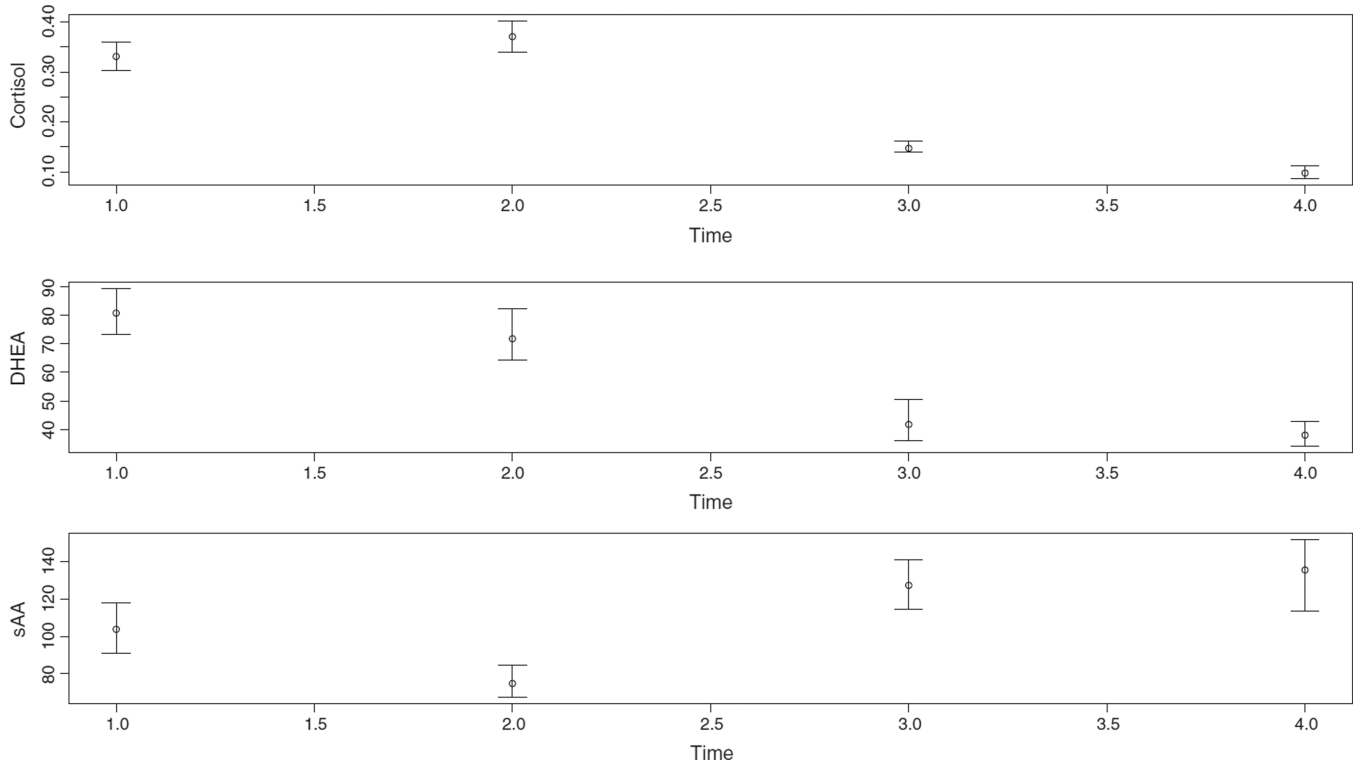

Fig. 2.
Medians at times 1, 2, 3 and 4.

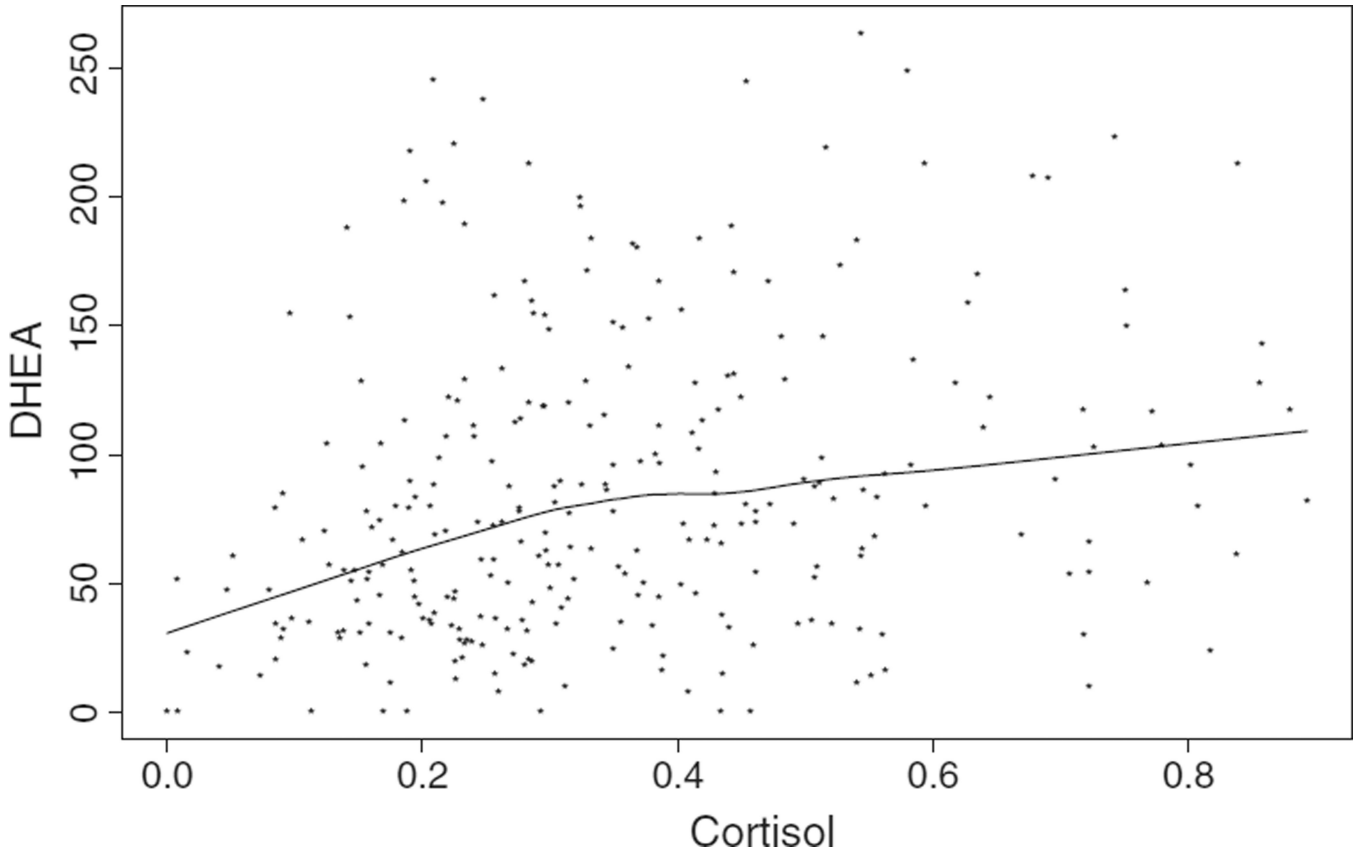

Fig. 3.
Regression line for predicting the typical DHEA level given cortisol upon awakening.

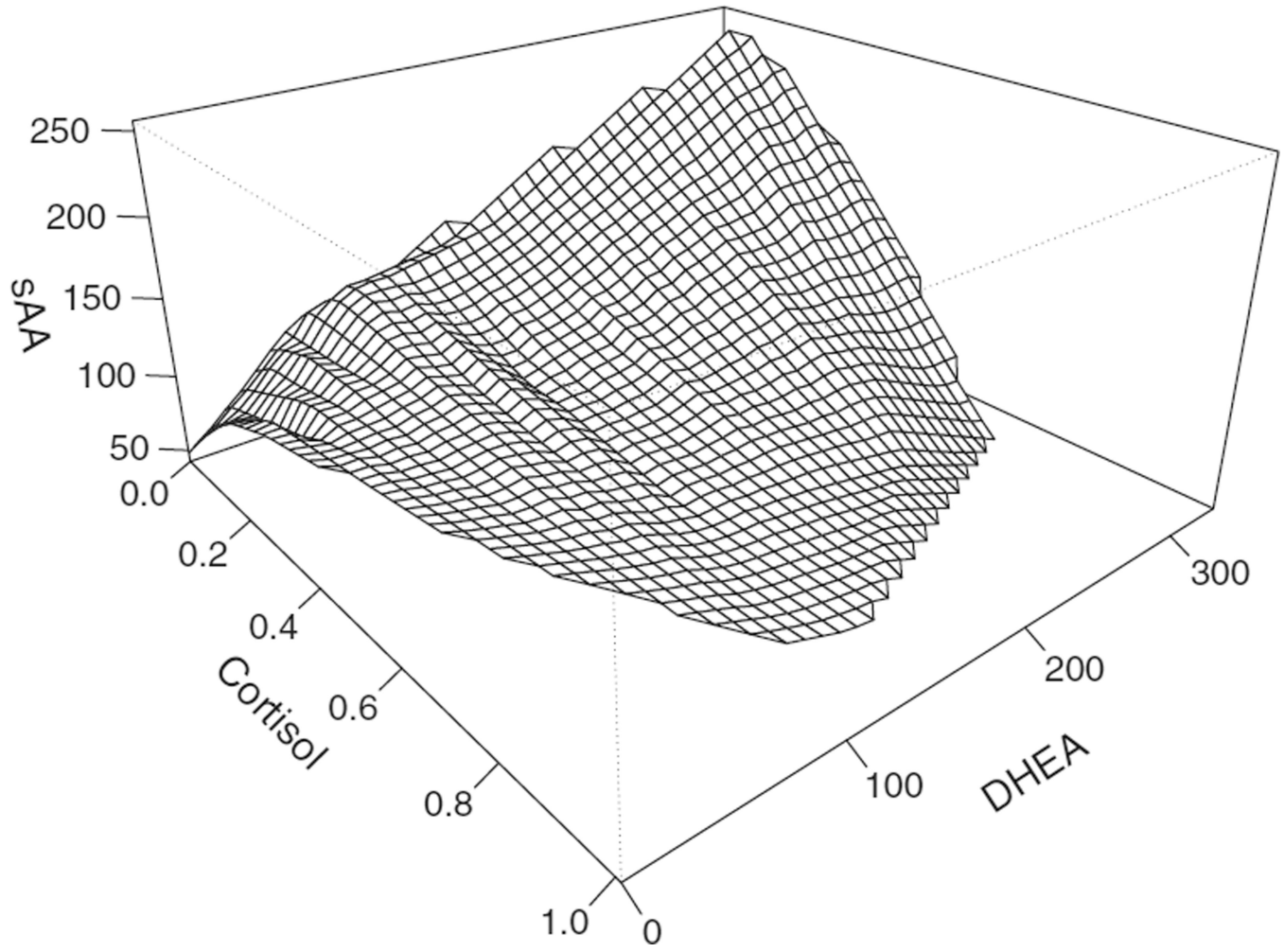

Fig. 4.

Regression surface for predicting the typical sAA level based cortisol and DHEA upon awakening.