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# FREE CORTISOL LEVELS AFTER AWAKENING: A RELIABLE BIOLOGICAL MARKER FOR THE ASSESSMENT OF ADRENOCORTICAL ACTIVITY

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## Summary

In three independent studies, free cortisol levels after morning awakening were repeatedly measured in children, adults and elderly subjects (total n= 152). Cortisol was assessed by sampling saliva at 10 or 15 minute intervals for 30-60 minutes, beginning at the time of awakening for two days (Study 1 and 2) or one (Study 3) day, respectively. In all three studies, free cortisol levels increased by 50-75% within the first 30 minutes after awakening in both sexes on all days. Premenopausal women consistently showed a stronger increase with a delayed peak after awakening compared to men on all days. In Study 2, there was a tendency for lower early morning free cortisol levels for women taking oral contraceptives (p=.10). Stability of the area under the curve (AUC) of the early morning free cortisol levels over the three (Study 1 and 2) or two (Study 3) days ranged between r=.39 and r=.67 (p<.001). Neither age, weight, nor smoking showed an effect on baseline or peak cortisol levels. Sleep duration, time of awakening and alcohol consumption also appeared to be unrelated to early morning free cortisol levels. From these data we conclude that in contrast to single assessments at fixed times, early morning cortisol levels can be a reliable biological marker for the individual's adrenocortical activity when measured repeatedly with strict reference to the time of awakening.

In search of an easy-to-assess index of adrenocortical status, measurement of single cortisol levels is frequently performed in the early morning hours. Adhering to laboratory routine and the assumption that circadian peak levels are observed at this time, blood or saliva is usually sampled between 0800 and 0900 hrs for analysis of total or free cortisol (1-3). The resulting hormone values show large individual variation with normal values for total cortisol ranging between 165-690 nmol/l and for free cortisol ranging between 5-23 nmol/l. Due to this large variability, there is a significant overlap of morning cortisol levels between healthy individuals and patients with adrenal insufficiency or Cushing's disease (4) which limits the diagnostic value of this measure. Moreover, the

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intraindividual stability of cortisol levels obtained between 0800 and 0900 hrs is rather low (5-6) suggesting that the measurement of unstimulated cortisol levels is of limited use during that time period.

One reason for inconsistent and unspecific results obtained with early morning cortisol levels could be that blood or saliva samples are usually obtained from individuals and patients at predefined times without reference to the individual time of awakening. Recently, evidence has accumulated that waking up in the morning is followed by an activation of the pituitary-adrenal axis with brief ACTH and cortisol pulses in the majority of subjects investigated (7-9) which is discussed as episodic secretion that serves as a synchronizer for the circadian rhythm of the HPA-axis (10). While these studies strongly suggest that cortisol levels change in response to awakening, they were not performed to study this phenomenon systematically with respect to it's potential diagnostic value.

The present paper is the first report of the time course and intraindividual stability of the free cortisol response to awakening over days and weeks. Furthermore, it documents the influence of gender, age, smoking and use of estrogen-containing medication on this endocrine response.

## Methods

SUBJECTS AND GENERAL EXPERIMENTAL OUTLINE
  In three separate studies, a total of 152 subjects were investigated for early morning free cortisol levels after awakening. The groups consisted of 21 boys and 21 girls in Study 1, 35 men and 35 women in Study 2, and 20 men and 20 women in Study 3. Subjects differed significantly in age between the three studies to test age effects on the early morning free cortisol levels. The mean age of the volunteers was 11.16 ± 1.99 years (Study 1), 26.5 ± 6.31 years (Study 2), and 70.4 ± 5.72 years (Study 3), respectively. Subjects in Study 1 were children recruited through their parents by local newspapers. In Study 2 undergraduate and graduate students as well as nonacademic individuals were recruited by flyers in the University of Trier, or by direct contact (i.e., letters or personal contact). In Study 3, elderly subjects from the local community were recruited through announcement in local newspapers or by direct contact. Participants in Study 2 were not paid for participation. Subjects of studies 1 and 3 received a small monetary incentive after return of the study material.

All subjects were medication free except for use of oral contraceptives (OC) in Study 2. Since another aim of the study was to monitor a possible modulation of the early morning free cortisol levels by OC, women taking this medication were not excluded. It was not possible to balance the number of females with respect to OC medication. Out of the 35 females in Study 2, 13 subjects reported to use OC for at least 6 months. In Studies 1 and 3, female subjects did not use contraceptive medication. All subjects reported to be in good health. In all three studies, subjects had to fill in a health questionnaire once reporting all health complaints during the sampling period. No additional medical examination was performed to validate the subjects' self report.

SALIVA COLLECTION

Subjects sampled saliva for cortisol assessment on three (Study 1 and 2) or two days (Study 3). In Studies 1 and 3 subjects were instructed to sample saliva on consecutive days. A weekly interval between sampling was chosen in Study 2. Saliva was sampled with the Salivette sampling device (Sarstedt, Rommelsdorf, Germany).

Table 1 shows the number of subjects, mean age, number of days with cortisol measurement, interval between the measurements, and time of sampling obtained after awakening. Subjects were instructed to start sampling saliva immediately at the time of awakening and to stay in bed until the second saliva sample was obtained, i.e. 10 minutes (Study 1) or 15 minutes (Study 2 and 3) after awakening.

TABLE I

Overview of Saliva Sampling for Assessment of Early Morning Free Cortisol Levels in Three Independent Studies

| Study | Subjects | Mean Age | Sample Days | Interval between sampling days | Minutes After Awakening |
|-------|----------|----------|-------------|-------------------------------|-------------------------|
| 1 | 42 children | 12 | 3 | consecutive | 0, 10, 20, 30 |
| 2 | 70 adolescents | 26 | 3 | one week | 0, 15, 30, 60 |
| 3 | 40 elderly adults | 70 | 2 | consecutive | 0, 15, 30, 60 |

Subjects were further instructed to complete sampling before breakfast in order to avoid contamination of saliva with food or drinks, e.g. coffee or fruit juice. If impossible, subjects were told to wait at least half an hour before having breakfast. Also, subjects were told to thoroughly rinse their mouth with tap water before sampling saliva. In addition, subjects were asked to mark the salivettes which were sampled after breakfast in order to control for possible effects of saliva contamination. Furthermore, subjects were instructed not to brush their teeth before completing saliva sampling in order to avoid contamination of saliva with blood caused by micro-injuries in the oral cavity. After sampling, subjects stored the saliva samples in their freezer until completion of sampling and then returned the samples to the laboratory.

SOCIODEMOGRAPHIC ASSESSMENT

Information on sex, age and weight was obtained from each subject. If the subjects were smokers, they were asked to provide information about their smoking habits (i.e., how many cigarettes they usually smoke during the day) and detailed information during the sampling days (i.e., how many cigarettes they had smoked the night before the sampling day). On days of saliva sampling, subjects filled out a state questionnaire providing information on alcohol consumption (Studies 2 and 3), time of going to bed, total time slept, time of awakening, and self reports of health status and acute stress. Women reported information on their menstrual cycle and use of OC (Study 2 only).

CORTISOL ANALYSIS

Salivary cortisol was analyzed with a time-resolved immunoassay with fluorescence detection (DELFIA) as described in detail elsewhere (11). Intra- and interassay variability of the assay was less than 10 and 12%, respectively.

STATISTICAL ANALYSIS

Three-way analyses of variance (ANOVAs) with repeated measures (group by day by time) were computed to reveal possible effects of the different main effects (day, time, sex, OC use, smoking) on the early morning free cortisol levels. Adjustments of degrees of freedom were employed according to Greenhouse-Geisser where appropriate. Where ANOVA procedures revealed significant interactions between group variables and cortisol levels, Newman-Keuls *post hoc* tests were employed to test specific effects and significance levels. Also, in case of significant results, effect sizes were computed. In case of ANOVAs revealing no effects, the power of the respective test was calculated (12). In order to test effects of age and weight on the cortisol levels, areas under the curve (AUC) of the cortisol levels on the single days were computed. Since the interest in these studies was to assess both basal cortisol levels at the time of awakening and the increase after awakening, the computation of AUC included the basal cortisol levels. Then, Pearson correlations were calculated between age and weight of the subjects, and the single and AUC cortisol levels, respectively. For assessing the stability of the early morning free cortisol levels over time, Pearson correlations were computed between the AUC cortisol levels across the three (studies 1 and 2) or two (Study 3) days. In order to test effects of psychological parameters on early morning free cortisol levels, Pearson correlations were computed between the individual scores on the personality scales and the single and AUC cortisol levels.

## Results

ANOVA revealed a significant time effect on the early morning free cortisol levels after awakening on all days. The respective F-values for the main effect of awakening were 30.2 (Study 1), 59.18 (Study 2), and 17.02 (Study 3; all F with p < .0001). The time course of the early morning free cortisol levels was similar in all three studies: Within the first 30 minutes after awakening, free cortisol levels showed an 50-75% increase. The mean absolute increase was 5.63 nmol/l (Study 1), 9.26 nmol/l (Study 2), and 8.46 nmol/l (Study 3) across the three (Studies 1 and 2) and two (Study 3) days. After 30 minutes, free cortisol levels started to decrease and returned to baseline levels at about one hour after awakening. Figure 1 shows the mean cortisol levels within the first hour after awakening on three (Studies 1and 2) and two (Study 3) days.

No significant difference was observed between the baseline levels at the time of awakening or the mean absolute increase across the three studies. The effect sizes of the time effect on the early morning free cortisol levels varied between $f^2=.30$ to $f^2=1.27$, thus explaining between 23 and 56 percent of variability in the cortisol secretion during the first hour after awakening.

In Study 2, women showed significantly larger increases of the early morning free cortisol levels after awakening compared to men. In Study 1, there was a tendency towards larger increases in girls compared to boys. The respective F-values for the (time by sex) interaction were 2.06 (Study 1; p=.11), 3.11 (Study 2; p<.05). In Study 3, the F-value of the (time by sex) interaction was .65, p>.20. Figure 2 shows the early morning

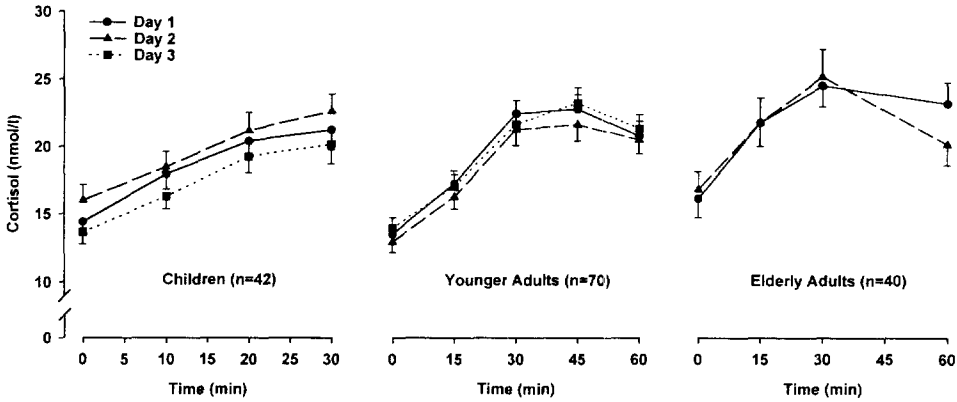free cortisol levels for all days of the three studies for men and women.



Fig. 1
Mean cortisol levels (± SE) after morning awakening in three independent studies. Subjects studied: children aged 7-14 (Study 1), adolescents aged 19-37 (Study 2), elderly adults aged 59-82 (Study 3)

The effect size for the time by sex effect on the early morning free cortisol levels in Study 2 was rather small ($f^2$=.05), thus explaining only 4 percent of variability in the early morning free cortisol levels. The respective baseline levels of cortisol at the time of awakening on the three days were comparable between men and women. Newman-Keuls *post hoc* tests indicated that the free cortisol levels were significantly different between men and women 30, 45 and 60 minutes after awakening. Thus, it was the decrease after the cortisol peak which discriminated best between sexes. Here, men tended to show more rapid decreases than women between 30 to 60 minutes after awakening. In Study 1, early morning free cortisol levels could only be sampled up to 30 minutes after awakening. Thus, here are only marginal differences in the cortisol levels between boys and girls.

Due to the subjects' age in Study 1 (children) and Study 3 (elderly subjects), women taking OC were present only in Study 2. In this study, a tendency for smaller cortisol increases after awakening occurred within the group of women under OC (F=2.16; p=.10 after Greenhouse Geisser adjustment of degrees of freedom). Figure 3 shows the early morning free cortisol levels in Study 2 for women with and without OC.

The effect sizes for the (time by OC) effect on the early morning free cortisol levels was $f^2$=.05, explaining 4% of variability in the early morning free cortisol levels. Thus, the effect of OC was comparable to the effect of gender on cortisol secretion after awakening. Newman-Keuls *post-hoc* tests indicated that differences were statistically significant at all time points, beginning at 15 minutes after awakening.

Smoking did not show a significant effect on the cortisol levels. In Study 2, the number of habitual smokers allowed statistical testing of possible effects of smoking on the early morning free cortisol secretion. Here, the ANOVA F-value for the main effect

of smoking was 1.59 (p >.20). The power of the test (presuming a small effect of $f^2 = .1$) with $\lambda = 12.5$ provided a 92.5 % chance of detecting possible effects of smoking in this sample.
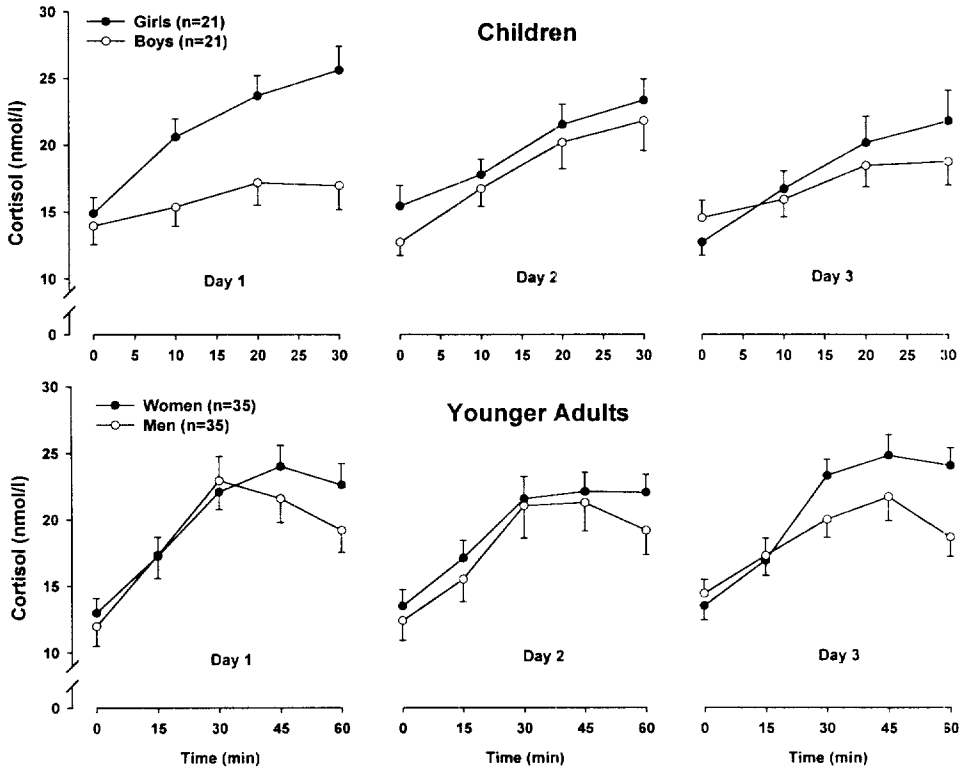


Fig. 2
Mean cortisol levels after awakening in three independent studies in women compared to men. For description of the subjects in the studies, refer to legend of Figure 1.

In order to test possible effects of time of awakening and total hours slept on the early morning free cortisol levels, Pearson correlations were calculated. All correlations failed to reach statistical significance at the 5% level, suggesting that cortisol secretion after awakening is rather independent of sleep duration or the time of awakening.

Next, the effects of age and weight on the early morning free cortisol levels were tested. In all three studies, correlations between the AUC cortisol levels of the single days with the respective age and weight data of the subjects failed to reach statistical significance, indicating no associations between age or weight and the respective cortisol levels after awakening. Pearson correlation between the mean cortisol increase (i.e., maximum cortisol levels during the first hour after awakening minus baseline

values) and the subjects' age over all three studies was r=.06, p>.20. Figure 4 shows the scatterplot for the increase of all subjects of the five studies and the subjects' age.
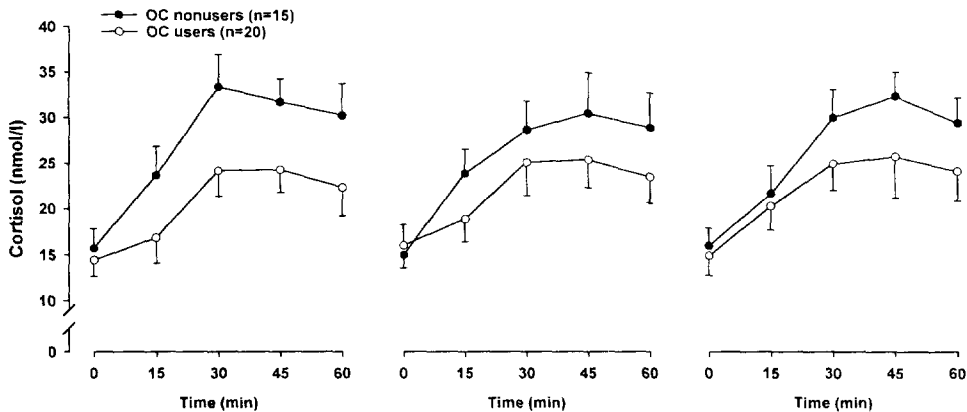


Fig. 3

Mean cortisol levels (± SE) after morning awakening in Study 2 in women using oral contraceptives compared with women using no contraceptive medication. For description of the subjects in the studies, refer to legend of Figure 1.

TABLE II

Pearson Correlations Between the AUC Cortisol Levels on the Single Days in Three Studies (for Subjects in the Study, Refer to Text)

| STUDY | | AUC1 | AUC2 |
|---|---|---|---|
| 1 | AUC2 | .39 | |
| | AUC3 | .67 | .57 |
| 2 | AUC2 | .49 | |
| | AUC3 | .42 | .65 |
| 3 | AUC2 | .58 | |

In order to test the stability of the early morning free cortisol secretion, AUC cortisol levels of the single days were intercorrelated. All correlations were significant at p<.05. They varied between r=.39 (AUC1-AUC2, Study 1) and r=.67 (AUC2-AUC3, Study 1). Thus, the explained variance for these correlations varied between 16 and 45 percent, indicating a moderate to high stability of AUC cortisol levels across days (Studies 1 and 3) and weeks (Study 2). Furthermore, this result indicates that stability of the early morning free cortisol levels after awakening is independent of the chosen time interval

between cortisol measurements. The intercorrelations between the single days of all three studies are shown in Table 2.
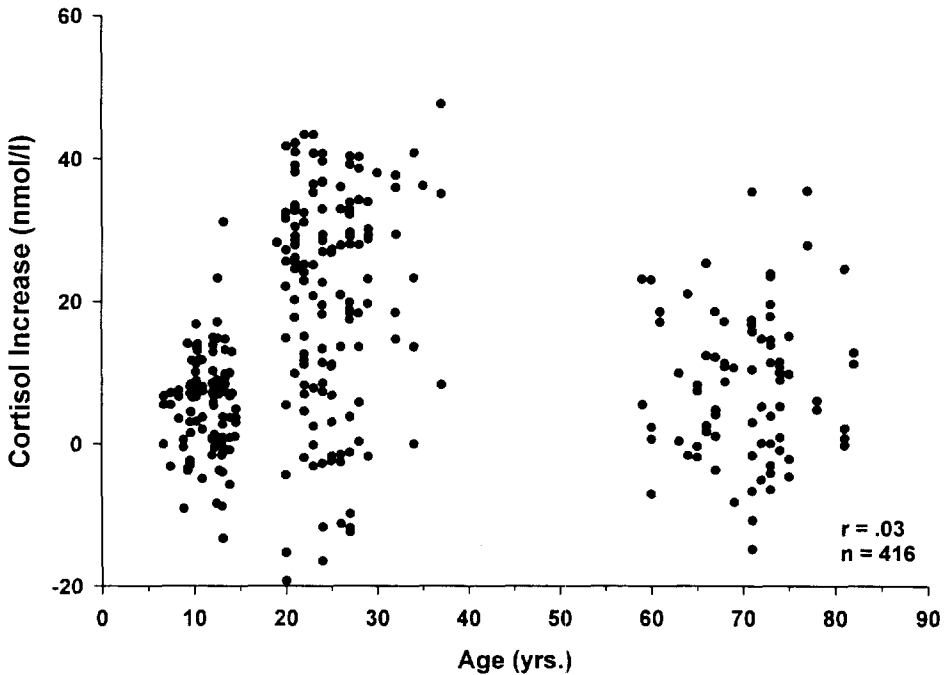


Fig. 4

Scatterplot of the cortisol increase after awakening (maximum cortisol levels minus baseline) versus age for 416 observations (Pearson correlation r=.06; n.s.)

## Discussion

The results suggest a potential usefulness of the early morning free cortisol response to awakening as a reliable biological marker for adrenocortical activity when measured repeatedly with strict reference to the time of awakening. The observed increase after awakening is consistent, shows good intraindividual stability across days and weeks and is able to differentiate between certain subgroups of healthy individuals. The stability of the three studies reported here varied between r=.39 and r=.70. Other studies reported stabilities over time for single cortisol assessment of r=.18 (5-6). Overall, these correlations indicate a surprisingly high intraindividual stability for a basal cortisol measure suggesting a potentially useful tool for assessment of individual differences. In fact, very recent results of this laboratory showed that in individuals characterized by chronic work overload, the morning cortisol rise is enhanced (13). On the other hand,

burn-out in teachers is associated with a blunted cortisol response to awakening (Pruessner et al., unpublished results).

A sex difference was observed in early morning free cortisol levels with women showing larger increases compared to men. For the first 30 minutes after awakening, increases of free cortisol levels in women were comparable with those of men. While women tended to show a further increase (or a delayed decrease), cortisol concentrations clearly decreased in men after the first half hour resulting in smaller AUCs compared to women. This sex difference was observed clearly in Study 2 on all days, indicating that this difference is consistent over time. In Study 1, where saliva sampling ended after 30 minutes, a tendency for further increases in women was observed. In Study 3, where only postmenopausal women were investigated, cortisol levels after awakening were comparable between men and women. A larger adrenocortical response to awakening in younger women appears to be in contrast to consistently smaller free cortisol increases following psychosocial stress compared to men (14). However, this may not be representative for a generalized lowered HPA responsiveness in women since physical exercise and CRH injections lead to similar or larger responses in premenopausal women (14-15). Moreover, an enhanced ACTH and cortisol response to h-CRH after dexamethasone pretreatment has been observed in elderly women (15) suggesting important modulatory effects of sex steroids on HPA responsiveness. This has been recently supported by findings of this laboratory. Men showed an exaggerated cortisol response to stress after a 2 day treatment with estradiol constantly delivered by a skin patch (16).

Small but consistent differences in free cortisol morning levels were also found between women using oral contraceptives (OC) compared to age-matched nonusers. From similar levels immediately after awakening, OC users showed lower free cortisol increases compared to medication-free women. Again, this effect was shown to be stable over time as indicated by the repeated assessment over three days. A blunted early morning free cortisol response in OC users compared to nonusers is in agreement with lower cortisol responses to psychosocial stress (17) or physical exercise (18-19) in women using OC medication. Current studies of this laboratory investigate the potential role of elevated corticosteroid-binding globulin levels in OC users in blunting the free cortisol response to awakening, psychosocial stress and pharmacological stimulation of the HPA axis. Numerous studies employing basal cortisol assessments in the morning failed to show any of these group differences (20-23).

The AUC cortisol levels were independent of the subject's age or weight. Moreover, the morning cortisol increase after awakening appears to be independent of the time of awakening, total time slept or alcohol consumption the night before. Future studies will need to investigate the cause of this cortisol response. Since subjects were asked to remain in a supine position for at least 15 minutes after awakening, it can be ruled out that the reported findings simply reflect an orthostatic response. In fact, there is no difference in the cortisol response to awakening whether subjects get up immediately or stay in bed for 15 minutes (unpublished data). Furthermore, it is unlikely that rising cortisol levels are a wake-up stimulus for the individual and that the reported rise is just a continuation of the cortisol increase. In additional experiments no differences in cortisol responses were observed when subjects were allowed to sleep as long as they desired without a wake-up alarm or when waking was induced using an alarm clock (unpublished data). This finding is in line with results from another laboratory (9,25). The cortisol increase after awakening could, however, represent the HPA axis response to a

centrally driven input designed to provide the organism with sufficient energy to shift from a resting to an active state. The teleological speculation is in part supported by data of Späth-Schwalbe et al. and Van Cauter et al. (9-10, 25), and awaits experimental proof in future studies.

In conclusion, waking up in the morning is a potent stimulus for the HPA axis resulting in a surprisingly stable increase of free cortisol levels over days and weeks. The present results suggest that a repeated measurement of cortisol responses in the morning could provide an important, easy-to-employ, and reliable tool in psychobiology and psychosomatic medicine when applied with strict reference to the time of awakening.

## Acknowledgements

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