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ORIGINAL ARTICLE

Age-related changes in hypothalamic–pituitary–adrenal axis activity in patients with manifest arterial disease

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Abstract Reports on age-related changes of hypothalamic–pituitary–adrenal (HPA) axis activity are equivocal. In addition, subtle changes in HPA axis activity are associated with cardiovascular risk factors. This study evaluates the effect of age in a large sample of patients with arterial disease on several parts of the circadian rhythm of the HPA axis. Within the Second Manifestations of Arterial Disease-Magnetic Resonance (SMART-MR) study, a prospective cohort study among patients with manifest arterial disease, cross-sectional analyses were performed in 419 patients (age 63 ± 9 years). Circadian cortisol rhythm was assessed with six saliva samples, collected at awakening and 30, 45, and 60 min thereafter and at 10 and 11 pm. Furthermore, a low dose of dexamethasone (0.5 mg) was administered at 11 pm, and saliva was sampled the next morning to test the cortisol suppression. Linear regression analyses adjusted for sex, awakening time, workday, smoking, blood pressure, BMI, diabetes mellitus, and dyslipidemia showed that older age was associated with a blunted cortisol awakening response. Per year increase, the rise ($\beta = -0.15$ nmol/l; 95%CI −0.25 to −0.05) and diurnal pattern ($\beta = -0.14$ nmol/l; 95%CI −0.25 to −0.02) decreased. Furthermore, older age was associated with higher evening levels (β log transformed = 0.01; 95%CI 0.01–0.02) and higher mean cortisol after dexamethasone (β log transformed = 0.01; 95%CI 0.002–0.02). In patients with arterial disease, HPA axis activity showed reduced variability with older age, independent of cardiovascular risk factors.

Keywords HPA axis · Age · Salivary cortisol · Circadian rhythm · Dexamethasone suppression test

Introduction

The hypothalamus pituitary adrenal (HPA) axis a is part of the neuroendocrine system that controls reactions to stressors and is important for homeostasis. It has been suggested that HPA functioning may serve as an indicator of the long-term effect of physiologic response to stress on the body, also referred to as allostatic load [1]. With aging, the allostatic load accumulates and this may cause changes in the HPA axis functioning [2, 3]. There is increasing evidence that change in the HPA activity is associated with several age-related pathologies, such as cardiovascular disease and Alzheimer's disease [4–6].

Data about changes of HPA axis activity during aging show mixed findings. Some studies report an increased mean basal cortisol activity with aging [3, 7, 8], while others report basal cortisol concentrations to be unaltered in the elderly [9, 10], for review see [11].

Most of these studies were done in healthy individuals in a relatively restricted age range. Since an altered HPA axis activity has often been associated with age-related illnesses, selecting healthy subjects may have made it more difficult to observe age-related changes. In fact, one reason that healthy individuals remained healthy may be that their HPA axis better maintained its resiliency and feedback sensitivity [11, 12]. To date, information about age-related

This study is conducted for the SMART study group.

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changes of the basal circadian cortisol pattern in a non-healthy population is scarce.

In this study, we investigated the association between age and several aspects of the basal diurnal rhythm including the cortisol awakening response (CAR), evening levels, the diurnal pattern, and the dexamethasone suppression test (DST) in a large sample of patients with manifest arterial disease. In addition, since several studies have suggested that subtle changes in HPA axis activity are associated cardiovascular risk factors, such as hypertension, diabetes mellitus, and central obesity (for review see, [13]), we also examined the associations of these risk factors and HPA axis activity within this group of subjects in whom cardiovascular risk factors are well characterized.

Results

The mean age ± SD of the study sample ($n = 419$) was 62.8 ± 9.2 years, with a range of 33–83 years. The characteristics of the study sample are presented across three age groups (Table 1). Patients in the youngest age group (<55 years) more often had to work on the sampling day and were awake earlier. The patients in the oldest age group (>65 years) smoked less, but had a higher mean systolic blood pressure.

Figure 1 shows the cortisol curve with unadjusted mean cortisol levels throughout the day for the three age groups. Using ANOVA, the oldest age group showed an attenuated awakening response compared with the youngest age group, with a mean difference for the rise of −4.1 nmol/l (95% CI −6.1 to −2.1 nmol/l), and for the area under the curve to the ground (AUCg) of −1.8 nmol/l*h (95% CI −3.5 to −0.1 nmol/l*h), and they had higher levels in the evening (mean difference of 0.8 nmol/l; 95% CI 0.0–1.6 nmol/l). The diurnal pattern was smaller in the oldest age group, compared with the youngest age group (mean difference of −3.8 nmol/l; 95% CI −6.0 to −1.5 nmol/l). There were no differences between the age groups in cortisol levels after dexamethasone suppression.

Figure 2 presents the scatterplots of age (on the *x*-axis) with the different cortisol measures (on the *y*-axis). As can be seen, the rise (Fig. 2a) and diurnal pattern (Fig. 2d) were significantly lower with increasing age ($r = -0.22$; $P < 0.001$ and $r = -0.2$; $P < 0.001$, respectively), while with increasing age, evening levels (Fig. 2c) as well as mean cortisol level after dexamethasone suppression (Fig. 2e) were higher ($r = 0.21$; $P < 0.001$ and $r = 0.17$; $P = 0.001$). The AUCg (Fig. 2b) was not significantly correlated with age ($r = -0.88$, $P = 0.88$) .

We examined the age adjusted association of several cardiovascular risk factors with the indices of HPA axis activity (Table 2). Higher diastolic blood and systolic pressure were significantly associated with higher rise and higher AUCg in the morning and subjects with dyslipidemia had lower levels of cortisol after dexamethasone compared to subjects without dyslipidemia. Furthermore, women had higher levels of cortisol after the DST compared to men (β log transformed = 0.20 nmol/l; 95% CI 0.01–0.39). There were no significant associations between the other risk factors and the measures of HPA axis activity.

The results of the linear regression analysis of the association between age and different measurements of HPA axis activity (Table 3) show that increasing age was significantly associated with a lower rise ($\beta = -0.17$ nmol/l; 95% CI −0.25 to −0.10) and a reduced

Table 1 Baseline characteristics of the study sample according to age group

	<55 ($N = 71$)	55–65 ($N = 170$)	>65 ($N = 178$)	Total sample ($N = 419$)
Age (years)[a]	49.0 ± 4.4	59.7 ± 2.8	71.4 ± 4.7	62.8 ± 9.2
Sex (male)[b]	85	87	84	85
Awakening time (h)[a]	6:44 ± 1:12	7:14 ± 1:05	7:23 ± 0:58	7:13 ± 1:05
Workday collection[b]	68	43	15	35
Smoking[b]	44	23	10	21
Systolic blood pressure (mmHg)[a]	138 ± 18	143 ± 19	148 ± 20	144 ± 20
Diastolic blood pressure (mmHg)[a]	86 ± 11	84 ± 10	81 ± 11	83 ± 11
Cortisol at awakening (nmol/l)[a]	11.0 ± 4.7	12.2 ± 5.6	11.9 ± 5.1	11.9 ± 5.2
Cortisol after 30 min (nmol/l)[a]	20.2 ± 9.2	18.2 ± 7.2	17.2 ± 7.3	18.1 ± 7.6
Cortisol after 45 min (nmol/l)[a]	20.0 ± 9.2	17.7 ± 7.2	17.5 ± 8.0	18.0 ± 8.0
Cortisol after 60 min (nmol/l)[a]	18.4 ± 9.2	16.0 ± 7.7	16.3 ± 7.6	16.6 ± 8.0
Mean evening cortisol (nmol/l)[a]	3.3 ± 1.7	3.4 ± 1.8	4.2 ± 3.7	3.7 ± 2.8
Cortisol after dexamethasone (nmol/l)[c]	1.5 (1.0, 12.1)	1.4 (1.0, 1.9)	1.7 (1.2, 2.5)	1.5 (1.1, 2.1)

[a] Mean ± SD, [b] percentage, [c] median (25th percentile, 75th percentile)

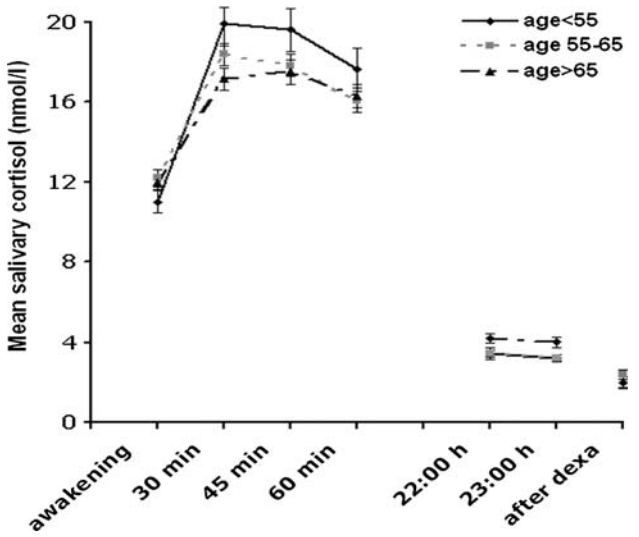

Fig. 1 Cortisol profiles (mean ± SEM) of all participants ($N = 419$) divided in three age groups. Shown are the awakening response, evening levels, and mean cortisol levels after ingestion of 0.5 mg dexamethasone

diurnal pattern ($\beta = -0.17$ nmol/l; 95% CI −0.26 to −0.08), and borderline significantly with a changed AUCg ($\beta = -0.06$ mol/l*h; 95% CI −0.12–0.01). Increasing age was also associated with higher levels in the evening and higher levels at awakening after dexamethasone. After adjustment for sex, awakening time, sampling on a workday, smoking and blood pressure, BMI, diabetes, and dyslipidemia, these results attenuated slightly (Table 3). When we added location of arterial disease [coronary artery disease, cerebrovascular disease, peripheral arterial disease, and abdominal aortic aneurysm (AAA)] to the model, the results did not materially change (data not shown).

One hundred and twenty three participants (29%) showed a rise in cortisol after awakening of less than 2.5 nmol/l. They were not significantly different from the rest of the sample with respect to sex, awakening time, smoking status, or blood pressure, on the bases of *t*-tests and Chi-square tests. However, there was a difference in age between the two groups with the non-responders being significantly older (65 vs. 62 years; $P = 0.007$). When we repeated the regression analyses after excluding these non-responders, the results did not materially change (Table 4).

Discussion

In this study, we examined the association between age and several aspects of HPA axis activity, determined by salivary cortisol, in a large sample of middle aged and elderly persons with manifest arterial disease. We observed that HPA axis activity showed reduced variability with older age in this population and that this age effect was independent of sex, cardiovascular risk factors, time of awakening, and workday. With older age, the awakening response as determined with the rise decreased, indicating a more blunted cortisol response after awakening. Evening cortisol levels were higher with increasing age, and the diurnal pattern was decreased. Furthermore, with increasing age HPA axis activity was less suppressed after dexamethasone. The area under the curve in the first hour after awakening was not significantly associated with age after adjusting for potential confounders.

In previous studies, a multitude of outcomes have been used to describe age-related changes in HPA axis activity. The evaluation of the HPA axis is complicated by the circadian cortisol pattern. After awakening, a rapid rise of 30–50% is observed and followed by a gradual decline during the day with low levels in the evening. Since different outcomes were examined in different study samples, it is difficult to compare the results of studies. In this study, we measured the cortisol response to awakening collected circadian cortisol profiles and determined the HPA feedback sensitivity following dexamethasone suppression in the same population, which allowed us to compare several aspects of HPA axis activity. To our knowledge, this is the first study determining the association between age and several outcomes used to describe the different aspects of circadian pattern of the HPA axis using salivary free cortisol in a non healthy population. Using a large study sample and of a non-healthy population increased the possibility to detect age-related changes in HPA axis activity [11]. The large sample size and the wide age range of subjects in this study, and the adjustment for a number of confounding factors made it possible to obtain relatively precise and valid estimates of the associations between age and HPA axis activity.

With respect to the awakening response studies have reported somewhat mixed findings. There is some evidence for a muted response after awakening with aging [3, 14]. In line with our findings are the results of a study that used 24-h cortisol measurements in plasma showing that the morning acrophase was associated with older age in normal elderly [3]. However, other studies did not find a relation between age and cortisol levels after awakening [15, 16]. These studies were done in healthy and relatively young subjects, which may have made it more difficult to detect age-related differences in HPA activity.

Since an altered HPA axis activity has often been associated with age-related illnesses, selecting only healthy subjects may have made it more difficult to observe age-related changes. If alterations in the HPA axis activity are associated with age-related diseases, selecting healthy individuals may blunt a studies' ability to detect changes because this population are those least likely to show age-related alternation in HPA axis activity. This is the reason

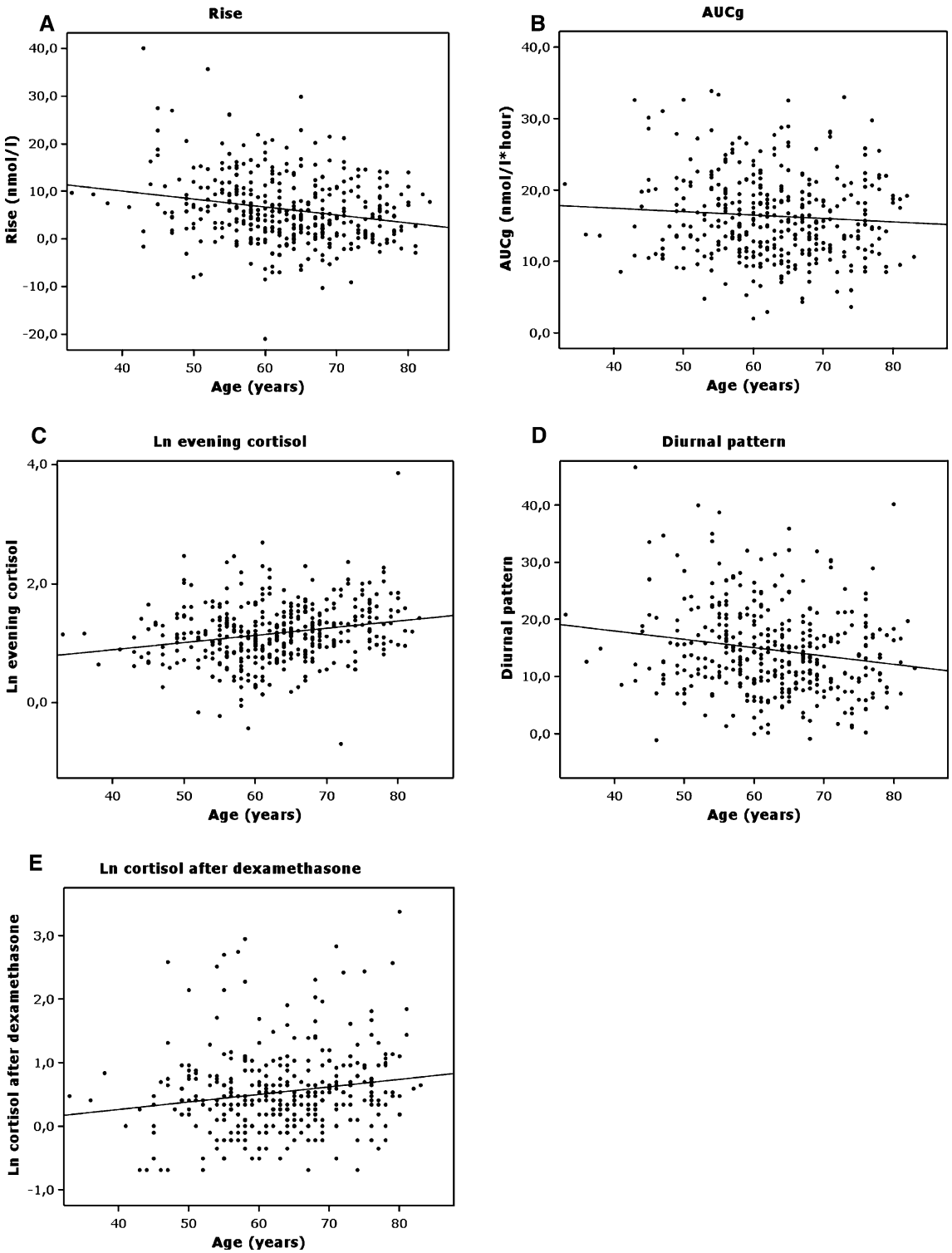

Fig. 2 Scatterplots representing the correlation between age and different cortisol measures: the rise (**a**), the area under the curve to the ground (**b**), the natural log transformed evening level (**c**), the diurnal pattern (**d**), and the natural log transformed cortisol level at awakening after the dexamethasone suppression test (**e**)

why we investigated this study population with a "challenged" body (i.e., one with vascular disease). However, this leaves the question if our results could be generalized to the general/or healthy population. It could be that healthy individuals remained healthy, because their HPA axis better maintained its resiliency and feedback

Table 2 Results of the linear regression analyses of the relation between age and different cortisol measures ($N = 419$)

	Rise (nmol/l)	AUCg (nmol/l*h)	Evening value (ln* nmol/l)	Diurnal pattern (nmol/l)	After dexa (ln* nmol/l)
Sex	−0.76 (−2.76–1.20)	0.92 (−0.80–2.63)	−0.003 (−0.14–0.13)	1.37 (−0.85–3.59)	0.20 (0.01–0.39)
Systolic blood pressure	0.05 (0.01–0.08)	0.03 (0.001–0.06)	−0.000 (−0.002–0.002)	0.03 (−0.01–0.08)	−0.002 (−0.01–0.002)
Diastolic blood pressure	0.06 (−0.01–0.13)	0.05 (−0.01–0.11)	−0.002 (−0.01–0.003)	0.07 (−0.01–0.14)	−0.01 (−0.01–0.000)
BMI	−0.01 (−0.19–0.17)	−0.12 (−0.28–0.04)	−0.004 (−0.02–0.01)	−0.12 (−0.32–0.09)	−0.01 (−0.02–0.02)
WHR	−0.15 (−3.51–3.20)	−0.68 (−3.59–2.24)	0.08 (−0.15–0.32)	−0.49 (−4.28–3.30)	−0.07 (−0.37–0.24)
Diabetes	0.30 (−1.45–2.05)	−0.65 (−2.20–0.89)	0.06 (−0.06–0.18)	−0.41 (−2.39–1.58)	−0.001 (−0.16–0.16)
Dyslipidemia	−0.42 (−2.17–1.34)	−0.09 (−1.64–1.45)	−0.09 (−0.20–0.031)	0.03 (−1.94–2.01)	−0.18 (−0.36 to −0.01)

AUCg Area under the curve with respect to the ground, *ln* natural log transformed, *BMI* body mass index, *WHR* waist hip ratio

Regression coefficients with 95% confidence interval, adjusted for age

Table 3 Results of the linear regression analyses of the relation between age and different cortisol measures ($N = 419$)

	Rise (nmol/l)	AUCg (nmol/l*h)	Evening value (ln* nmol/l)	Diurnal pattern (nmol/l)	After dexa (ln* nmol/l)
Model 1[a]	−0.17 (−0.25 to −0.10)	−0.06 (−0.12–0.01)	0.01 (0.01–0.02)	−0.17 (−0.26 to −0.08)	0.01 (0.01–0.02)
Model 2[b]	−0.15 (−0.25 to −0.06)	−0.04 (−0.13–0.04)	0.01 (0.01–0.02)	−0.15 (−0.26 to −0.04)	0.01 (0.002–0.02)

AUCg area under the curve with respect to the ground, *ln* natural log transformed

[a] Crude regression coefficients with 95% confidence interval

[b] Adjusted for sex, awakening time, workday, smoking, systolic, diastolic blood pressure, BMI, Diabetes mellitus and dyslipidemia

Table 4 Results of the linear regression analyses of the relation between age and different cortisol measures for responders[a] ($N = 296$)

	Rise (nmol/l)	AUCg (nmol/l)	Evening value (ln* nmol/l)	Diurnal pattern (nmol/l)	After dexa (ln* nmol/l)
Model 1[b]	−0.15 (−0.22 to −0.07)	−0.03 (−0.12–0.05)	0.01 (0.01–0.02)	−0.15 (−0.25 to −0.04)	0.01 (0.01–0.02)
Model 2[c]	−0.12 (−0.22 to −0.03)	−0.02 (−0.12–0.08)	0.01 (0.01–0.02)	−0.13 (−0.26 to −0.001)	0.01 (0.002–0.02)

AUCg area under the curve with respect to the ground, *ln* natural log transformed

[a] Responders are defined as subjects with an increase of cortisol after awakening of 2.5 nmol/l or more

[b] Crude regression coefficients with 95% confidence interval

[c] Adjusted for sex, awakening time, workday, smoking, systolic, diastolic blood pressure, BMI, Diabetes mellitus and dyslipidemia

sensitivity [11, 12]. To date, information about age-related changes of the basal circadian cortisol pattern in a non-healthy population is scarce.

In our study, we adjusted for known confounding factors, such as awakening time or sampling on workday. Also, we tried to maximize compliance by giving clear instructions and having the patient record the sampling time. Still, we cannot exclude the possibility of non-adherence to the sampling protocol in some persons. Especially persons without a rise within the first hour after awaking may have been non-compliant. We found that persons with a rise lower than 2.5 nmol/l were significantly older than persons with a clear awakening response. It could thus be argued that the blunted awakening response that we observed with older age may be due to non-compliance in the older age group. However, when we excluded persons with a rise lower than 2.5 nmol/l, the results were quite similar after adjustment for covariates. It is thus possible that absence of a clear cortisol response in the morning is the result of an intrinsic aberrant awakening response. Since an absent response after awakening is also found in several pathologies, it may well be that with increasing age there are more non-responders with a physiologic explanation, instead of merely non-adherence to the sampling protocol [17, 18].

Consistent with other reports using blood sampling, although not all [10], we found higher mean post-dexamethasone cortisol levels in the older subjects [19, 20]. These findings suggest that with aging the ability to suppress glucocorticoids is diminished. There is evidence that the sensitivity of the HPA axis to negative feedback by cortisol is decreased in older people [21]. It has been

suggested that this loss of sensitivity may account for elevated cortisol secretion in the evening [22]. Our finding of higher evening cortisol concentrations with increasing age is consistent with this explanation.

Our observation of elevated evening values and an attenuated awakening response, which resulted in a diminished diurnal pattern, could be indicative of a reduced range of HPA axis activity. This loss of variability has also been reported by several other authors and is related to several risk factors for cardiovascular disease, type 2 diabetes, and stroke, and is also seen in patients with dementia [7, 23, 24]. It has been hypothesized that loss of variability is a consequence of repeated or chronic challenges of the HPA axis by several stress factors, which may make an individual more susceptible to disease and it may be an important factor to the onset of frailty in the elderly [1, 25, 26].

It is thus possible that in our population, altered HPA axis functioning contributed to the development of arterial disease. In our study, the higher levels in the morning were associated with higher systolic and diastolic blood pressure but not with other cardiovascular risk factors. These results are in line with earlier studies that examined the relationship between cortisol and blood pressure [27, 28]. Since our study sample consisted of patients with arterial disease, we cannot exclude the possibility that the observed age-related changes are the result of the arterial disease. We tried to minimize this possibility by adjusting for cardiovascular risk factors, but the sample had arterial disease nonetheless. As a result, we do not know to what extent our findings could be generalized to the general population.

Given the cross-sectional nature, it is hard to disentangle whether the observed association between age and HPA axis activity in our study population is a cause or a consequence of the health status. However, the results of this study are in line with the idea that the allostatic load due to a lifetime exposure to stressors accumulates with age and may cause a loss in resiliency of the HPA axis functioning [1].

In summary, in this population of patients with arterial disease, we observed that HPA axis activity showed a reduced variability with aging. Older age was associated with a blunted awakening cortisol response, higher levels of cortisol in the evening, decreased diurnal variability, and diminished suppression after dexamethasone.

Materials and methods

SMART-MR study

We used cross-sectional data from the Second Manifestations of Arterial Disease-Magnetic Resonance (SMART-MR) study, a prospective cohort study within the SMART study [29] on brain changes on MRI in patients with manifest arterial disease. Between May 2001 and December 2005, all patients were eligible for the SMART-MR study if they were newly referred to the University Medical Center Utrecht with coronary artery disease, cerebrovascular disease, peripheral disease or an abdominal aortic aneurysm (AAA), and had no MR contraindications. During a one-day visit to our medical center, an MRI of the brain was performed, in addition to a physical examination, ultrasonography of the carotid arteries, and blood and urine sampling. Risk factors, medical history, and functioning were assessed with questionnaires that the patients filled in before their visit to the medical center. Methods for baseline examinations have been described extensively in a previous study [30]. In total, 1309 patients (mean age 58 $\pm$ 10 years; 80% male) were included in the SMART-MR study. The SMART study and SMART-MR study were approved by the ethics committee of our institution and written informed consent was obtained from all participants.

Starting in January 2006, all the patients who are still alive have been invited for a second MR of the brain and other follow-up examinations, including assessment of risk factors and cognitive testing. In addition, measurements of HPA axis activation were added. In 2006 and 2007, a total of 499 patients (mean age 62 $\pm$ 9 years; 85% male) received follow-up examinations.

HPA axis activity

HPA axis activity was assessed at home by six measurements of cortisol in saliva over a period of 24 h to obtain the circadian rhythm. The saliva was collected using cotton dental rolls (Salivette, Startstedt). Participants were instructed to refrain from smoking, drinking caffeine, eating, or cleaning their teeth at least 30 min before collecting a saliva sample. On day 1, participants were instructed to take the first sample immediately after awakening while still lying in bed, and to take the second, third, and fourth samples after 30, 45, and 60 min, respectively. Samples 5 and 6 were collected at 10 and 11 pm, respectively. In addition, participants were asked to take 0.5 mg of dexamethasone orally after their sixth saliva sample, and to sample their saliva again the next day directly after awaking. They were instructed to start sampling a few days before their visit to the hospital and to store their saliva samples in their freezers, until the day of the visit. At the lab, the saliva samples were centrifuged at 3000 rpm for 10 min and then stored at -80°C until assayed. The cortisol in saliva was measured without extraction using an in house competitive radio-immunoassay employing a polyclonal anticortisol-antibody (K7348). [1,2-^{3}H(N)]-Hydrocortisone (NET185, NEN—DUPONT, Dreiech, Germany) was used

as a tracer. The lower limit of detection was 0.5 nmol/l, and inter-assay variation was 9% at 3 nmol/l and 5% at 23 nmol/l. Intra-assay variation was 4%.

HPA axis activity and its circadian pattern were quantified in several ways. First, the CAR, which is the immediate increase of cortisol in the hour after awakening, was computed as the area under the cortisol curve to the ground with respect to zero (AUCg) and as the rise. The AUCg was calculated by multiplying the levels of the four morning cortisol samples by the time interval between sampling points in minutes [31]. The rise was defined as the difference between waking and the S_{30} values. The AUCg provides information about overall levels of cortisol post-awakening, whereas the rise represents the dynamic of the change in concentration of cortisol during the awakening response. Second, evening levels of cortisol were defined as the average of the saliva samples taken at 10 and 11 pm. Third, the diurnal pattern was calculated as the difference between the sample at 30 min after awakening, and the average evening value indicates the cortisol decline during the day. Finally, as an indicator of suppression of the HPA axis, the cortisol value was taken at awakening the morning after the ingestion of the dexamethasone.

Assessment of covariates

Participants were asked to record the time at which each saliva sample was taken and to complete a questionnaire, providing information on smoking status, time of awakening and whether they had to work on the sampling day. All these factors are considered as potential confounders (for review see [32]). During the patient's visit to the hospital, an overnight fasting venous blood sample was drawn to determine glucose and lipid levels. Height and weight were measured, and the body mass index (BMI) was calculated (kg/m^2). Waist circumference was measured halfway between the lower rib and the iliac crest, and hip circumference was measured at the level of the greater trochanter. Blood pressure was assessed by measuring three times systolic and diastolic blood pressures (mmHg) with a sphygmomanometer when the patient was seated. The average of the three measurements was taken for both diastolic and systolic blood pressures. Diabetes mellitus (DM) was defined as glucose $\geq$7.0 mmol/l or when the patient reported having DM or using oral antidiabetic drugs or insulin. Dyslipidemia was defined as total cholesterol >5.0 mmol/l, low-density lipoprotein cholesterol >3.2 mmol/l, or self reported use of lipid lowering drugs.

Study sample

Of the 499 patients who were examined in 2006 and 2007, the data of cortisol levels of 42 patients were missing, due to development of the sampling protocol, refusal of patients to collect saliva and because some received saliva samples were lost. One patient did not collect sufficient amounts of saliva. Furthermore, there were six patients who did not adhere to the sampling times described in the sampling protocol. These seven patients were excluded from the study sample. Since we performed complete case analysis, an additional 31 patients were lost from analysis, because these subjects had missing values on one or more covariates. The excluded patients were somewhat younger (60 vs. 63 years), but other patient characteristics were comparable between the excluded patients and the 419 patients available for analysis. For the analysis of dexamethasone, 36 patients had missing data because they refrained from taking the dexamethasone or did not deliver enough saliva for analysis of cortisol after dexamethasone.

Data analysis

As the cortisol data of evening values and the mean cortisol after ingestion of dexamethasone were highly skewed, we used a natural log transformation to normalize the distribution. First, using ANOVA, we calculated the unadjusted mean levels of free cortisol for the measures of HPA axis activity across three age groups (<55 years, 55–65 years, and >65 years). Second, Pearson's product moment correlation coefficients were used to calculate correlations between age and measures of cortisol. Third, since dysregulation of the HPA axis has been implicated with several cardiovascular risk factors, age-adjusted linear regression was used to examine the associations of the risk factors: sex, blood pressure, BMI, waist hip ratio (WHR), diabetes mellitus, and hyperlipidemia with the different measures of HPA axis activity as dependent variable. Fourth, linear regression analysis was used to determine the relation between age (on a continuous scale) and the measures of HPA axis activity. In the first model, univariable associations between age and HPA axis outcomes were estimated, and in the second model adjustments were made for sex, awakening time, workday (yes vs. no), smoking, BMI, hyperlipidemia (yes vs. no), diabetes mellitus (yes vs. no), and blood pressure. Finally, we additionally adjusted for location of vascular disease (cerebrovascular disease yes vs. no; cardiovascular disease yes vs. no; peripheral disease yes vs. no; and AAA yes vs. no). We repeated the regression analyses after excluding patients who showed no clear cortisol response after awakening, because these "non-responders" may not have complied with the protocol. An increase of cortisol after awakening of less than 2.5 nmol/l was defined as non-response [16]. Assumptions of all models were tested by residual analyses. In all analyses, the 95% confidence intervals are given.

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Declaration of interest The authors report no conflicts of interest.

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