Associations between hypothalamic–pituitary–adrenal axis function and peak bone mass at 20 years of age in a birth cohort

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Abbreviations: AUC, area under the curve; BMI, body mass index; BMD, bone mineral content; BMD, bone mineral density; CV, coefficient of variation; DXA, dual-energy x-ray absorptiometry; HPA, hypothalamic-pituitary-adrenal; TSST, Trier Social Stress Test

Abstract

In older adults, high-normal circulating cortisol levels are associated with lower bone mass, but relationships between hypothalamic-pituitary-adrenal axis function and peak bone mass in young adults have not been examined. We studied 411 male and 390 female participants in the Western Australia Pregnancy Cohort (Raine) Study. At 18 years of age, participants underwent a Trier Social Stress Test (TSST) with measurement of plasma and salivary cortisol at baseline and at multiple time points after stress. Cortisol responses were classified as anticipatory responder (significant fall in cortisol during the test), reactive responder (significant increase) or non-responder. At 20 years, total body bone mineral content (BMC) and density (BMD) were measured by DXA. In males, after adjustment for weight, height (for BMC and bone area only), alcohol and smoking, there was a significant inverse relationship between both plasma and salivary cortisol measured at baseline in the TSST and each of BMC and BMD, such that each additional 10% of salivary cortisol was associated with reductions of 6.9 g (95% CI -11.7, -2.2) in BMC, and 1.8 mg/cm^2 (95% CI -3.3, -0.4) in BMD. Males classified as anticipatory responders in the TSST had 3.2% lower BMC (adjusted mean ± SE: 3131 ± 28 vs. 3233 ± 18 g, P=0.006) and 2.5% lower BMD (1108 ± 9 vs. 1136 ± 6 mg/cm^2, P=0.022) than reactive responders. In females, there were no significant relationships between baseline cortisol or TSST responses and BMC or BMD in covariate-adjusted analyses. We conclude that in young males (but not females), higher circulating cortisol at the baseline of the stress test and an anticipatory responder pattern on the TSST are associated with lower total body bone mass.

Introduction

It is well-recognized that glucocorticoid treatment is a risk factor for osteoporosis (1), and excessive endogenous cortisol production in Cushing's syndrome leads to significant reduction in bone mineral density (BMD) and increased fracture risk (2). Less is known on the effects of endogenous cortisol within the physiological range on bone health, but there is evidence from epidemiological studies in older adults that high-normal cortisol levels may be associated with lower BMD (3-6) or increased rate of bone loss (3, 7).

Analysis of the relationships between endogenous cortisol and bone health is challenging because of the complexity of hypothalamic–pituitary–adrenal **(**HPA) axis physiology, including diurnal variation, stress responses, and interactions between obesity and cortisol secretion and metabolism. In previous studies of older adults, a range of measures of HPA axis function have been used, including integrated 24-hour cortisol level and trough cortisol concentration (3), morning and evening salivary cortisol levels (4), post-dexamethasone cortisol level (5, 6), and peak plasma cortisol following tetracosactrin stimulation (7). The Trier Social Stress Test (TSST), a psychosocial stress protocol, has been shown to reliably and consistently produce HPA axis stimulation and elicit the highest endocrine responses of any laboratory stressor (8, 9), therefore may be more physiologically relevant than pharmacological stressors in the evaluation of HPA axis activity. However, the association between the HPA axis response to TSST and bone density has not been studied. In addition, there have been no studies examining the relationships between HPA axis function and peak bone mass in young adults. This is potentially important, as attainment of optimal peak bone mass in early adult life is considered the best protection against osteoporosis in later life (10).

In the Western Australia Pregnancy Cohort Study, a well-characterized community-based cohort study, HPA axis function was evaluated by the Trier Social Stress Test at late adolescence (18 years of age). In this analysis, we examined relationships between HPA function at late adolescence and bone mass measured at 20 years of age in participants, when peak bone mass is generally attained (11).

Subjects and methods

Subjects

The study participants were from the Western Australian Pregnancy Cohort (Raine) Study, which recruited 2,900 pregnant women from the public antenatal clinic at King Edward Memorial Hospital and surrounding private clinics in Perth, Western Australia between May 1989 and November 1991, and has subsequently followed the offspring as a birth cohort study. Inclusion criteria were a gestational age between 16 and 20 weeks, English language skills sufficient to understand the study demands, an expectation to deliver at King Edward Memorial Hospital, and an intention to remain in Western Australia to enable future follow-up of their child (12). Compared with the general Western Australian population, the Raine cohort at birth was characterized by higher proportions of high-risk births and fathers employed in managerial and professional positions, but comparison of participants remaining in the study at the 14-year follow-up suggested attrition resulted in a cohort comparable with the general population (13). Of the 2,868 children born, 1,306 participated in the physical examination component of the 20-year cohort follow-up, of whom 1,183 had valid whole body dual-energy x-ray absorptiometry (DXA) scans (14). Of these, 872 underwent the TSST at 18 years. Excluding 24 participants who were taking medications such as exogenous steroids, neuroactive or anti-depressant medications at the time of TSST, 33 participants who did not complete the test or had diurnal disturbances (worked night shifts or had little sleep), and 14 participants displayed unusual patterns which could not be categorized (it was unclear if this was due to physiological or technical reasons), data from 411 males and 390 females were included in this analysis (**Figure 1**). The study at both 18 and 20 years of age was approved by the Human Research Ethics Committee of University of Western Australia. Written informed consent was obtained from each participant.

Trier Social Stress Test (TSST)

A TSST was conducted at the 18-year follow-up visit. Participants were instructed to refrain from physical exercise, smoking, medication, eating and drinking anything besides water for one hour before the test, which was conducted in the afternoon between 1200h and 1600h. This time of day was selected in order to minimise the effect of HPA axis circadian rhythmicity on the results. Prior to the test, participants completed a questionnaire regarding current medication use, smoking habits, and oral contraceptive use. An intravenous cannula was inserted into a forearm vein under local anaesthesia for blood sampling, and participants then rested for 45 minutes before starting the test. The test itself took 15 minutes to complete and consisted of a free speech interview and an arithmetic task facing a non-responsive audience and a dummy camera according to established protocols (15, 16). Blood samples were taken for plasma total cortisol levels just prior to the test (baseline, 0 minutes), after completing the test (15 minutes) and then at 25, 35, 45, 60, 75 and 105 minutes. Saliva samples were collected using Salivette collection devices (Sarstedt, Germany) from all participants at 0, 15, 35 and 105 minutes. In addition, for the 153 participants (99 females) who elected not to have blood sampling, saliva were collected at all eight time points as for the schedule for blood collection. No formal assessment for gum disease was made, but bleeding from gum disease would significantly increase salivary cortisol levels from contamination with blood, resulting in a clear outlier and exclusion from the analysis. Time zero was reported as the start of the TSST as per the standard protocol (15). All biological samples were kept on ice during the test, then centrifuged, aliquoted and frozen at -80ºC until assayed. For plasma and salivary cortisol, area under the curve above baseline with respect to increase (AUC_I) was calculated using the trapezoidal rule (17); this parameter has been shown by principal components analysis to emphasize changes in cortisol profile over time (18).

TSST responses were categorised into three patterns: reactive responder, anticipatory responder and non-responder, in alignment with previous reports of distinct response patterns in the literature (19, 20). The primary parameter used for stress pattern determination was the plasma total cortisol measured at eight regularly spaced time points. Additional information was derived from the secondary parameter of salivary cortisol. For participants who only had salivary cortisol measured, the stress pattern was determined from the salivary cortisol recorded at eight time points. A set of criteria for the grouping of TSST patterns was developed and refined from examination of the literature and response data, and the method has been described in detail elsewhere (21). In brief, reactive responders were defined as having an increase in cortisol from baseline of greater than twice of the CV of the cortisol assays (22), in this case 13.2% for the plasma and 9.04% for the salivary cortisol assay. Anticipatory responders were defined as exhibiting a drop from baseline within the first 60 minutes of greater than twice the CV plus an estimation of afternoon cortisol changes due to diurnal variation (21) . Non-responders did not show reactive or anticipatory responses. The proportion of participants in each response pattern did not differ significantly between those with saliva samples only and those with both blood and salivary samples (males $P = 0.450$; females $P = 0.239$).

Laboratory assays

Plasma total cortisol and salivary free cortisol were quantified using the GammaCoat^{TM} ^{125}I cortisol radioimmunoassay kit (DiaSorin, Stillwater, MN, USA). All the samples from the same participant were analysed within the same assay. The inter-assay CV for plasma and salivary cortisol were 6.6% and 4.5% respectively, and the intra-assay CV were <10%. The sensitivity of the assay was 5.8 nM for total plasma cortisol and 0.3 nM for salivary cortisol.

126 The assay is highly specific for cortisol with minimal cross-reactivity (~1%) with other
127 endogenous corticosteroids. All samples were assayed in duplicate against an appropriate
128 standard curve and were repeated with additional dilutions, where required.
129
130 ***Whole body DXA***
131 Whole body DXA was performed at the 20-year follow-up visit using on a Norland XR-36
132 densitometer (Norland Medical Systems, Inc., Fort Atkinson, WI, USA), according to
133 manufacturer-recommended procedures. Analysis of scans was performed using built-in
134 machine software (version 4.3.0) and all analyses were checked by one researcher (JM) for
135 consistency. The analysis provided estimates of whole body bone mineral content (BMC) (g),
136 bone area (cm^2) and areal BMD (mg/cm^2). The densitometer had a variation in precision of
137 <2.0% for the measured site at standard speed.
138
139 ***Other assessments***
140 Weight and height were measured with subjects dressed in light clothes at 18 and 20 years.
141 Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$. Information on smoking
142 habit at 18 and 20 years, and contraceptive use (females only) at 18 years was collected using
143 questionnaires. A validated semi-quantitative food frequency questionnaire from the Cancer
144 Council Victoria (23) was used to assess dietary intake including calcium and alcohol intake
145 at 20 years. Physical activity level at 20 years was assessed using the International Physical
146 Activity Questionnaire (IPAQ), and categorised as low, medium and high according to the
147 IPAQ scoring protocol (24).
148
149 ***Data analysis***

Variables are presented as mean (SD) for each sex unless otherwise stated. The normality of continuous variables was checked through the construction of histograms. Baseline plasma and salivary cortisol were logarithmically transformed prior to analysis as they exhibited a skewed distribution. Comparisons between males and females were made by Student t-test or chi-square test as appropriate. The associations of baseline cortisol, as well as AUC_I and TSST response patterns (derived from data at multiple time points) with bone measures were evaluated in males and females separately. Correlation coefficients between baseline cortisol and bone measures were calculated using Pearson's correlation analysis in males, and in females the partial correlations were calculated accounting for oral contraceptive use. These associations were further evaluated using linear regression models with bone measures as dependent variables, baseline cortisol or AUC_I as predictor variables, and incorporating the following covariates: weight and alcohol consumption at 20 years, and smoking at 18 and 20 years, which were chosen based on evidence of the influence of lifestyle factors on bone health (25). Age was not adjusted for in the models due to the narrow age range of the study participants. Height at 20 years was adjusted in the models for total body BMC and bone area as it is highly correlated with these two variables. Results were further adjusted for calcium intake and physical activity at 20 years for participants with these data available. To account for the inter-correlation between predictor variables, the semi-partial R^2 for each predictor variable was calculated to estimate the proportion of the variance associated uniquely with each predictor. Collinearity was tested in each regression model, and a variance inflation factor (VIF) value larger than 10 was considered as showing the existence of collinearity or near collinearity (26). Collinearity was not observed in any of the models. Comparisons between the three TSST response categories were made by analysis of variance (ANOVA) with Tukey's HSD post hoc test or chi-square test in each sex. Comparisons between bone measures in these three groups were made by analysis of covariance (ANCOVA) adjusting

for the covariates listed above with Bonferroni post hoc test. There were significant interactions between sex and predictor variables (HPA measures) on the outcome measures and we elected to analyse data from males and females separately. Women taking oral contraceptives had higher plasma cortisol concentrations and lower total body BMD than women not taking them, but the interaction terms for oral contraceptive use and predictor variables were not significant for any outcome measures, indicating that the relationships between HPA measures and bone were not significantly altered by contraceptive use. We thus included oral contraceptive treatment as a covariate in the analysis in females, but did not analyse subgroups of women according to contraceptive use. Statistical significance level was set at $P < 0.05$ (two-tailed). All analyses were performed using IBM SPSS (version 21, IBM, Chicago, IL, USA).

Results

Characteristics of participants

The mean age (SD) of participants at the time of the TSST was 18.3 (0.3) years and at time of DXA scanning was 20.0 (0.4) years. At 18 and 20 years, there were no significant differences between male and female participants in age and BMI, but males were taller and heavier and more likely to smoke (**Table 1 & 2**). At 18 years, the baseline mean plasma cortisol level was lower in males than in females, but there was no significant sex difference in peak plasma cortisol. Salivary cortisol was significantly higher in males compared with females for both baseline and peak levels. The higher baseline total plasma cortisol levels in females were mainly due to the high levels in those on oral contraceptive (565.9 ± 265.6 nM), as estrogen increases corticosteroid-binding globulin levels (27). Males actually had higher total plasma cortisol levels than females not on oral contraceptive (375.5 ± 177.1 vs 309.6 ± 141.8 nM, $P<0.001$), and higher salivary cortisol levels than females either on oral contraceptive or not (15.3 ± 10.3 vs 13.2 ± 10.4 and 12.3 ± 7.3 nM, both $P < 0.05$). There were no significant sex differences in AUC_I for either plasma or salivary cortisol (Table 1). At 20 years, compared to females, males had significantly higher calcium and alcohol intake, physical activity level, and total body BMC, bone area and BMD (Table 2).

TSST baseline cortisol and bone mass

In males, plasma and salivary cortisol measured at TSST baseline each showed a weak negative correlation with total body BMC (r: -0.135 and -0.124, respectively, both $P<0.05$) and BMD (r: -0.146 and -0.132, respectively, both $P<0.01$) but no significant correlation with bone area. In addition, the AUC_I for both plasma and salivary cortisol had a significant positive correlation with total body BMD (r: 0.106 and 0.126, respectively, both $P<0.05$). In females, there was a weak negative partial correlation (after accounting for oral contraceptive

use) between baseline plasma cortisol and each of total body BMC (r: -0.133, $P<0.05$) and bone area (r: -0.187, $P<0.01$) but not BMD. There were no significant correlations between salivary cortisol or AUC_I for either plasma or salivary cortisol and any bone measures in females.

In males, after adjustment for relevant covariates, the inverse relationships between baseline plasma and salivary cortisol and each of total body BMC and BMD, and the positive relationship between AUC_I for salivary cortisol and total body BMD remained significant (**Table 3)**. In addition, AUC_I for salivary cortisol showed a positive relationship with total body BMC after the adjustment of covariates (Table 3). In females, only the negative association between total plasma cortisol and total body bone area remained significant after covariate adjustment. Based on the regression coefficients of log-transformed predictor variables, we estimate that in males, each additional 10% of baseline plasma cortisol was associated with reductions of 6.8 g (95% CI -13.2, -0.3) in total body BMC and 2.2 mg/cm^2 (95% CI -4.2, -0.2) in total body BMD, whereas each additional 10% of salivary cortisol was associated with reductions of 6.9 g (95% CI -11.7, -2.2) in total body BMC, and 1.8 mg/cm^2 (95% CI -3.3, -0.4) in total body BMD. In females, each additional 10% increase in total plasma cortisol at baseline was associated with a reduction of 3.1 cm^2 (95% CI -5.8, -0.3) in total body bone area. When physical activity level and calcium intake were further adjusted for in subjects with these data available (298 males and 337 females), results were essentially unchanged, except the associations between AUC_I for total plasma cortisol and total body BMC and BMD became significant in males (β 3.5, 95% CI 0.4-6.7, $P = 0.029$ and β 1.0, 95% CI 0.02-1.9, $P = 0.046$, respectively).

In males, baseline total plasma and salivary cortisol uniquely associated with 0.8% to 1.7% of the variance in total body BMC and BMD (semi-partial R^2 0.008 – 0.017), which is higher than the variance uniquely associated with calcium intake for total body BMC (0.6-0.8%). Physical activity level was not a significant predictor of bone measures in any models.

Analyses by TSST response pattern

The percentages of participants in the three TSST response groups – reactive responder, anticipatory responder and non-responder – were 63.3, 24.6 and 12.1% for males and 51.3, 29.5 and 19.2% for females, respectively, a significant sex difference (P=0.001). In both genders, there were no significant differences between the three TSST response patterns in anthropometric and lifestyle characteristics, except for female non-responders, who had significantly lower body weight and BMI than reactive responder group at both age 18 and 20 years (Tables 1 & 2). As expected, in both genders baseline plasma and salivary cortisol were significantly higher in anticipatory responder than the other two groups, whereas peak plasma cortisol was higher in reactive responders and anticipatory responders than non-responders. The AUC_I for both plasma and salivary cortisol were highest in reactive responders, and lowest in anticipatory responders (Table 1).

In males, in the unadjusted analysis, total body BMC and BMD were significantly lower in anticipatory responders compared to reactive responders, with no significant differences between non-responders and the other groups (Table 2). After adjustment for relevant covariates, the differences remained significant, such that total body BMC was 3.2% lower (P=0.006) and BMD 2.5% lower (P=0.022) in the anticipatory responder group than in the reactive responder group (**Table 4**).

In females, in the unadjusted analysis, non-responders had significantly lower total body BMD than reactive responders (Table 2), but the significance did not remain after covariate adjustment (Table 4). In both males and females, results were essentially unchanged after further adjustment for physical activity level and calcium intake in subjects with these data available (data not shown).

Discussion

In this study of young adults, we found that in males, plasma and salivary cortisol measured at 18 years of age at the baseline of a well-validated stress test had a negative association with total body BMC and BMD measured at 20 years, independent of body weight, alcohol intake, smoking status, calcium intake and physical activity level. Males classified as anticipatory responders (characterized by high pre-test cortisol levels which fell after stress) had 3.2% lower total body BMC and 2.5% lower total body BMD than reactive responders (who had lower pre-test cortisol levels which increased after stress). In females, however, there were no significant relationships between cortisol or TSST responses and BMC or BMD in covariate-adjusted analyses. These results suggest that in males (but not females), endogenous cortisol secretion and its response to stressful stimuli may be one of the factors contributing to peak bone mass. Since a 5% difference in BMD is associated with a 20% difference in the risk of osteoporotic fracture and a 50% difference in the risk of hip fracture (28), the magnitude of the differences observed may be clinically relevant, with implications for the fracture risk in later life.

An association between endogenous cortisol and peak bone mass is biologically plausible, since excess exogenous or endogenous glucocorticoids as in Cushing's syndrome leads to a reduction in bone mass and quality, and increased fracture risk (29). Our results are consistent with a small number of observational studies in older adults, in which high-normal glucocorticoid levels within the physiological range are associated with reduced bone density in cross-sectional analysis, and accelerated bone loss during follow-up (3-7). These studies have generally been interpreted as indicating that endogenous glucocorticoids contribute to age-related bone loss. Our study extends these findings to young adults and suggests that as well as contributing to bone loss in the elderly, endogenous glucocorticoid secretion and its

289 response to stressful stimuli may affect attainment of peak bone mass. Dual roles of
290 endogenous glucocorticoids during development and aging are biologically plausible, since
291 serum cortisol is a heritable trait (with heritability estimates of up to 60%) (30, 31), which
292 demonstrates considerable intra-individual reproducibility on repeated sampling over time
293 (32); thus an individual with high-normal cortisol levels in childhood is likely to have high-
294 normal levels in later life. Our study assessed total cortisol in plasma samples, where the
295 majority of cortisol is bound to cortisol binding globulin and albumin, and salivary cortisol
296 which reflects free cortisol. Biological activity may depend more upon free cortisol, as
297 concentrations of carrier proteins have been reported to vary between and within individuals
298 depending on factors including oestrogen status and posture (27, 33). These factors and the
299 greater number of subjects with salivary samples may explain the stronger association of
300 salivary cortisol with total body BMC and BMC compared with plasma total cortisol.
301
302 In the TSST, the majority of participants were classified as reactive responders,
303 demonstrating a dynamic HPA response to stress. Anticipatory responders appeared to be
304 "pre-stressed" before the TSST, with significantly higher baseline cortisol levels than reactive
305 responders and non-responders. We speculate that anticipatory responders have a subtle but
306 significant chronic excess in cortisol exposure compared with reactive responders, which may
307 contribute to the lower bone mass seen in males in this group, as increased circulating
308 glucocorticoids could cause the apoptosis of osteoblasts and reduce their activity and increase
309 the activity of osteoclasts (2). In contrast to the negative association between baseline cortisol
310 and bone mass, AUC_I for salivary cortisol during the TSST was positively associated with
311 BMC and BMD in men, and there were similar trends (although not statistically significant)
312 for plasma cortisol. This positive relationship between a measure of cortisol secretion and
313 bone mass appears paradoxical. The normal HPA axis is in a state of dynamic equilibrium

and different patterns of glucocorticoid presentation exert different responses which are tissue specific (34), and it is conceivable that normal HPA activity in some way exerts a positive influence on bone health. Consistent with this, in a study of premenopausal women, there was a positive relationship between salivary peak cortisol after awakening and bone mass assessed by calcaneal ultrasound (35). It is also possible, however, that higher AUC_I and higher bone mass are each indicators of better general health, with no causal relationship between the two. In non-responders, pre-test cortisol levels were comparable to reactive responders but AUC_I was significantly lower. Despite these differences in cortisol profile, bone mass did not differ significantly between non-responders and the other two groups. The reason for this is uncertain and warrants further study.

While it would be difficult to modify endogenous cortisol, optimising early life factors might benefit HPA axis function, and thus the development of peak bone mass. In animal studies, protein restriction during mid- and late pregnancy is associated with reduced methylation of key CpG-rich islands in the promoter region of the glucocorticoid receptor (GR) gene, resulting in increased GR expression and features of hypercortisolism (36). Maternal stress during pregnancy is known to influence the developing HPA axis in the foetus (37).

In females, baseline plasma cortisol showed a negative relationship with total body BMC and bone area in the unadjusted analysis, but after adjustment for covariates, only the negative correlation with bone area remained significant. Non-responders had significantly lower total body BMD than reactive responders in unadjusted analysis, but that was no longer significant after the low body weight of non-responders had been accounted for. The basis for the sex difference in the results of our study is not clear, but it is consistent with other reports of sex differences in the cortisol-bone relationship. For example, in mice, endogenous

339 glucocorticoid signalling is required for normal bone structure, growth, and strength in
340 females but not males (38). In a study of Chinese people, a common variant in GR was
341 associated with extreme BMD in men but not women (39), and in a cohort study of healthy
342 older individuals, an association between baseline urinary free cortisol and incident fracture
343 was more apparent in men than in women (40). *In vitro*, cortisol has sex-specific effects on
344 activity of the enzyme aromatase P450 (which converts testosterone to estradiol), stimulating
345 aromatase in subcutaneous preadipocytes prepared from women, but inhibiting its activity in
346 preadipocytes prepared from men (41). Since estradiol has a positive effect on bone mass in
347 both men and women (42), this provides a possible basis for a sex difference in the effect of
348 cortisol on bone, although it is important to note that effects of cortisol on aromatase activity
349 in bone tissue have not been studied.
350
351 In the present study, we used TSST baseline plasma and salivary cortisol levels, AUC_I during
352 the test, and TSST response pattern to evaluate the relationship. This has the advantage of
353 providing a pre-test measure of HPA axis function, increase of cortisol in response to stress
354 and a response to a social stress. Other strengths of the study include the large sample size,
355 detailed analysis of the TSST response patterns, and assessment of bone mass at skeletal
356 maturity. Our study also has limitations. Firstly, its observational, cross-sectional nature
357 means we cannot assume that the relationships between cortisol and bone are causal.
358 Although we adjusted for several important confounding variables, the significant
359 associations observed may still be due to potential residual or uncontrolled confounders.
360 Secondly, most of the participants were Caucasian, and the study findings may not be
361 applicable to other ethnic groups. Thirdly, the DXA scans were performed two years later
362 after the TSST. However, since intra-individual variation in plasma cortisol is less than inter-
363 individual variation over time, and biological effects of endogenous cortisol on bone are

likely to be long term, this should not be a significant confounder. Longitudinal studies (1.5-6 years) with repeated measures of cortisol levels and circadian rhythm (43) and cortisol reactivity at the laboratory challenge (44) in children and adolescence have shown relative stability of HPA activity across time. Another limitation is that we did not measure BMD at fracture relevant sites such as spine and hip. Nevertheless, previous studies have shown a close relationship between BMD measures of total body, lumbar spine and hip (45), and the value of total body BMD in predicting hip fracture (46).

In conclusion, in young males, but not females, high circulating cortisol at the baseline of a social stress test, smaller area under the curve during the test and therefore an anticipatory responder TSST pattern are associated with lower total body BMC and BMD. Understanding the role of endogenous cortisol levels on bone physiology may be of value in promoting optimal peak bone mass development in young adults.

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Table 1 Characteristics of male and female participants at 18 years of age according to TSST response pattern

	Male				Female			
	All (n = 411)	Reactive responder (n = 260)	Anticipatory responder (n = 101)	Non-responder (n = 50)	All (n = 390)	Reactive responder (n = 200)	Anticipatory responder (n = 115)	Non-responder (n = 75)
Age, years	18.3 (0.3)	18.2 (0.2)	18.3 (0.3)	18.3 (0.3)	18.3 (0.3)	18.3 (0.3)	18.3 (0.2)	18.3 (0.3)
Height, cm	178.4 (7.0)*	178.9 (6.9)	177.8 (7.0)	177.0 (7.5)	165.9 (6.0)	165.5 (5.6)	166.4 (6.8)	166.5 (6.0)
Weight, kg	74.3 (13.6)*	74.7 (13.8)	73.1 (12.0)	74.0 (15.4)	64.1 (12.5)	65.6 (13.4)	63.9 (11.8)	60.3 (10.0)[a]
BMI, kg	23.3 (3.9)	23.3 (4.0)	23.1 (3.5)	23.6 (4.3)	23.3 (4.4)	23.9 (4.8)	23.1 (4.0)	21.8 (3.5)[a]
Current smoker, %	14.4**	11.9	17.8	20.0	8.7	5.5	11.3	13.3
Oral contraceptive use, %	-	-	-	-	41.0	38.5	41.7	46.7
BL plasma cortisol, nM	375.5 (177.1)**	324.4 (144.9)	527.1 (188.3)[a,b]	346.5 (140.0)	410.0 (235.4)	363.5 (210.4)	525.6 (268.4)[a,b]	364.6 (189.2)
BL salivary cortisol, nM	15.4 (10.3)*	12.4 (7.1)	23.1 (13.2)[a,b]	14.7 (9.1)	12.8 (9.3)	10.4 (5.6)	18.5 (12.8)[a,b]	10.6 (6.5)
Peak plasma cortisol, nM	518.0 (200.5)	534.1 (197.1)	546.5 (204.1)[b]	386.4 (159.7)[a]	524.5 (257.3)	563.9 (257.0)	538.2 (274.4)[b]	413.6 (200.4)[a]
Peak salivary cortisol, nM	20.2 (11.4)*	19.7 (10.4)	23.6 (13.3)[a,b]	15.7 (10.2)	16.5 (10.2)	16.8 (8.8)	18.8 (12.7)[b]	11.9 (7.4)[a]
AUC_I plasma cortisol	-987 (11430)	6003 (8571)	-15161 (7086)[a,b]	-3420 (3306)[a]	-249 (11768)	6318 (9666)	-12968 (7100)[a,b]	-2184 (3313)[a]
AUC_I salivary cortisol	-115.3 (626.2)	226.6 (513.4)	-900.4 (831.1)[a,b]	-257.0 (273.7)[a]	-110.7 (754.3)	200.1 (452.5)	-659.5 (678.7)[a,b]	-121.8 (285.0)[a]

Values are means (SD) unless otherwise stated. For plasma cortisol (BL, peak and AUC_I), n = 357 and 291 for males and females, respectively. *P < 0.05, **P ≤ 0.001 compared with female (Student t-test or chi-square test); [a]P < 0.05 compared with reactive responders, [b]P < 0.05 compared with non-responders in the same sex (ANOVA with Tukey post hoc test). BL: baseline; AUC_I: Area under the curve with respect to increase.

Table 2 Characteristics of male and female participants at 20 years according to TSST response pattern

	Male				Female			
	All (n = 411)	Reactive responder (n = 260)	Anticipatory responder (n = 101)	Non-responder (n = 50)	All (n = 390)	Reactive responder (n = 200)	Anticipatory responder (n = 115)	Non-responder (n = 75)
Age, years	20.1 (0.4)	20.0 (0.4)	20.1 (0.4)	20.0 (0.5)	20.0 (0.4)	20.0 (0.4)	20.0 (0.4)	20.1 (0.5)
Height, cm	178.8 (7.0)*	179.2 (6.9)	178.7 (7.3)	177.1 (7.0)	166.0 (6.2)	165.9 (6.0)	166.3 (6.8)	166.0 (6.1)
Weight, kg	76.8 (13.8)*	77.4 (14.1)	75.7 (12.9)	75.6 (14.1)	65.2 (12.7)	66.3 (13.5)	65.3 (11.8)	61.9 (11.7)[a]
BMI, kg/m^2	24.0 (3.9)	24.1 (3.9)	23.7 (3.7)	24.1 (4.0)	23.6 (4.6)	24.1 (4.9)	23.6 (4.0)	22.5 (4.4)[a]
Calcium intake, mg/day	1018 (370)*	1015 (386)	1033 (347)	1001 (339)	834 (306)	830 (300)	819 (278)	864 (360)
Current smoker, %	18.0**	16.2	18.8	26.0	11.3	10.5	12.2	12.0
Alcohol intake ≥3 units/d, %	12.9*	12.3	12.9	16.0	3.3	4.0	2.6	2.7
Physical activity, % Low	6.7	7.3	4.5	8.1	12.6	12.4	12.0	13.9
Medium	30.9	28.8	31.8	40.5	53.4	54.3	52.0	52.8
High	62.4*	63.9	63.6	51.4	34.1	33.3	36.0	33.3
Total body BMC, g	3201 (425)**	3251 (424)	3111 (413)[a]	3123 (424)	2689 (330)	2712 (340)	2693 (319)	2623 (316)
Total body bone area, cm^2	2834 (189)**	2849 (186)	2814 (192)	2798 (193)	2634 (179)	2633 (176)	2646 (184)	2623 (180)
Total body BMD, mg/cm^2	1127 (107)**	1139 (107)	1103 (102)[a]	1113 (107)	1019 (83)	1028 (85)	1016 (79)	999 (85)[a]

Values are means (SD) unless otherwise stated. For calcium intake n = 362 and 365; for physical activity level n = 330 and 358 for male and female, respectively. *$P < 0.05$, **$P \leq 0.001$ compared to female (Student t-test or chi-square test); [a]$P < 0.05$ compared to reactive responders in the same sex (ANOVA with Tukey post hoc test).

Table 3 Regression coefficients for baseline cortisol and total body bone measures

	Model with Log [Total plasma cortisol, nM]		**Model with Log [salivary cortisol, nM]**		**Model with [AUC_I total plasma cortisol/1000]**		**Model with [AUC_I salivary cortisol/1000]**	
	β (95% CI)	P	β (95% CI)	P	β (95% CI)	P	β (95% CI)	P
Male								
BMC, g	-71.2 (-139.0, -3.4)	**0.040**	-72.8 (-122.8, -22.9)	**0.004**	2.4 (-0.2, 5.0)	0.068	52.0 (15.4, 88.7)	**0.005**
Bone area, cm^2	-4.9 (-29.1, 19.3)	0.690	-13.4 (-31.2, 4.5)	0.141	0.2 (-0.7, 1.1)	0.660	1.9 (-11.3, 15.0)	0.781
BMD, mg/cm^2	-23.0 (-43.9, -2.0)	**0.032**	-19.4 (-35.1, -3.8)	**0.015**	0.8 (-0.04, 1.6)	0.061	17.3 (5.9, 28.7)	**0.003**
Female								
BMC, g	-28.0 (-80.9, 24.9)	0.298	1.3 (-34.1, 36.7)	0.944	1.2 (-0.9, 3.2)	0.255	7.0 (-23.8, 37.8)	0.655
Bone area, cm^2	-32.1 (-60.8, -3.5)	**0.028**	-4.2 (-23.2, 14.9)	0.666	0.4 (-0.7, 1.6)	0.423	1.1 (-15.5, 17.7)	0.896
BMD, mg/cm^2	1.7 (-16.3, 19.8)	0.850	1.6 (-10.7, 13.9)	0.802	0.2 (-0.5, 0.9)	0.505	1.7 (-9.0, 12.4)	0.758

AUC_I: Area under the curve with respect to increase. Other independent variables included in the models are weight, height (for BMC and bone area only) and alcohol consumption at 20 years, smoking at 18 and 20 years, and oral contraceptive use at 18 years (female only).

Table 4 Total body bone measures according to TSST response pattern

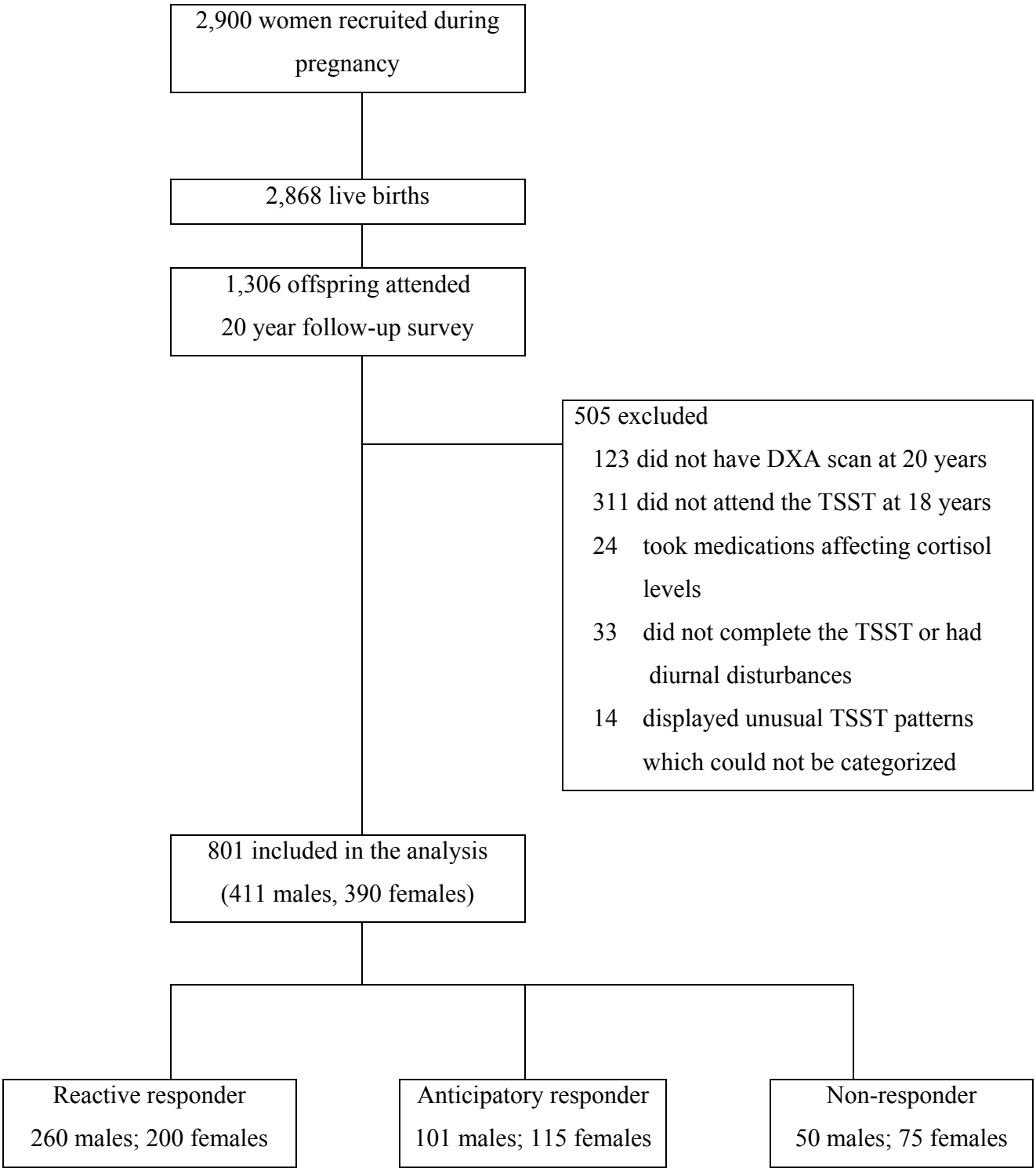
	Reactive responder	**Anticipatory responder**	**Non-responder**	**P**
Male				
N	260	101	50	
Bone mineral content, g	3233 (18)	3131 (28)[a]	3177 (40)	**0.007**
Bone area, cm^2	2840 (6)	2818 (10)	2834 (14)	0.196
Bone mineral density, mg/cm^2	1136 (6)	1108 (9)[a]	1118 (13)	**0.020**
Female				
N	200	115	75	
Bone mineral content, g	2697 (14)	2686 (18)	2675 (22)	0.683
Bone area, cm^2	2629 (7)	2640 (10)	2641 (12)	0.529
Bone mineral density, mg/cm^2	1023 (5)	1016 (6)	1012 (8)	0.400

Values are adjusted means (SEM), calculated using analysis of covariance (ANCOVA) adjusted for weight, height (for BMC and bone area only) and alcohol consumption at 20 years, smoking at 18 and 20 years, and oral contraceptive use at 18 years (female only). [a]$P < 0.05$ compared with reactive responders in the same sex (ANCOVA with Bonferroni post hoc test).

Figure legends

Figure 1 Participant disposition chart showing how the study population was derived. DXA, dual-energy x-ray absorptiometry; TSST, Trier Social Stress Test

Figure 1


2,900 women recruited during pregnancy
2,868 live births
1,306 offspring attended 20 year follow-up survey
505 excluded
123 did not have DXA scan at 20 years
311 did not attend the TSST at 18 years
24 took medications affecting cortisol levels
33 did not complete the TSST or had diurnal disturbances
14 displayed unusual TSST patterns which could not be categorized
801 included in the analysis (411 males, 390 females)
Reactive responder
260 males; 200 females
Anticipatory responder
101 males; 115 females
Non-responder
50 males; 75 females