RESEARCH

Higher urinary cortisol levels associate with increased cardiovascular risk

Andrea V Haas[1], Paul N Hopkins[2], Nancy J Brown[3], Luminita H Pojoga[1], Jonathan S Williams[1], Gail K Adler[1] and **Gordon H Williams[1]**

[1]Division of Endocrinology, Diabetes and Hypertension, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA
[2]Cardiovascular Genetics, Department of Internal Medicine, University of Utah School of Medicine, Salt Lake City, Utah, USA
[3]Vanderbilt University Medical Center, Nashville, Tennessee, USA

Correspondence should be addressed to A V Haas: ahaas2@bwh.harvard.edu

Abstract

There are conflicting data on whether variations of physiologic cortisol levels associated with cardiovascular risk. We hypothesize that prior discordant findings are related to problems associated with varying sample size, techniques for assessing cardiovascular risk and failure to adequately account for environmental factors. To address these issues, we utilized a large sample size, selected the Framingham risk score to compute cardiovascular risk and performed the study in a highly controlled setting. We had two main objectives: determine whether higher, yet physiologic, cortisol levels associated with increased cardiovascular risk and determine whether caveolin-1 (rs926198) risk allele carriers associated with increased cardiovascular risk. This was a cross-sectional study of 574 non-diabetic individuals who completed a common protocol. Data collection included fasting blood samples, blood pressure measurements and a 24-h urine-free cortisol collection. Five hundred seventeen of these participants also completed caveolin-1 genotyping. Subjects were classified as belonging to either the low-mode or high-mode urine-free cortisol groups, based on the bimodal distribution of urine-free cortisol. In multivariate analysis, Framingham risk score was statistically higher in the high-mode cortisol group (10.22 (mean) ± 0.43 (s.e.m.)) compared to the low-mode cortisol group (7.73 ± 0.34), $P < 0.001$. Framingham risk score was also statistically higher in the caveolin-1 risk allele carriers (8.91 ± 0.37) compared to caveolin-1 non-risk allele carriers (7.59 ± 0.48), $P = 0.034$. Overall, the estimated effect on Framingham risk score of carrying the caveolin-1 risk allele was 1.33 ± 0.61, $P = 0.029$. Both urinary cortisol and caveolin-1 risk allele status are independent predictors of Framingham risk score.

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Introduction

It is well established that individuals with Cushing's syndrome, a disease of excess glucocorticoid production, have increased rates of hypertension, obesity, type 2 diabetes and cardiovascular (CV) events (1). Likewise, individuals with incidentally discovered adrenal masses and biochemical work-up consistent with subclinical Cushing's syndrome also have increased cardiometabolic risk (2, 3, 4, 5, 6). Even among individuals with

biochemical work-up consistent with non-functional adrenal tumors, there is a two-fold elevated risk of diabetes, raising the possibility that 'non-functional' adrenal tumors may actually produce small, yet excess, amounts of glucocorticoids (7). Together, these studies implicate a role of both overt and non-overt hypercortisolism in the pathogenesis of cardiovascular disease (CVD).

However, much less is known about the CV effects of cortisol concentrations within the physiological range. While some studies have shown a link between higher cortisol levels and the presence of CV risk factors (8, 9, 10, 11, 12, 13), others have not (14, 15, 16, 17). The reasons for the observed discordance are uncertain, but could relate to sample size, methods used to assess CV risk and failure to adequately control for environmental factors. Most prior studies evaluated cortisol's effect on single risk factor analyses (e.g. obesity or glucose intolerance) rather than overall or composite CV risk. The Framingham risk score is a well validated and widely used prediction tool of 10-year CV events and death (18, 19). To our knowledge, the Framingham risk score has never been utilized to assess the relationship between cortisol and CV risk.

In addition, there is mounting evidence that CV risk is heritable (20). Members of our group have previously shown that a prevalent caveolin-1 (CAV1) gene variant (rs926198) is related to cardiovascular risk (insulin resistance and the metabolic syndrome) (21, 22). CAV1 is a scaffolding protein and is important in CV and kidney tissue signal transduction (22). Accordingly, we sought to determine whether there was a relationship between the CAV1 risk allele and increased cardiovascular risk as well as the possibility of an interaction between cortisol levels, CAV1 gene variants and CV risk (23, 24).

To address the potential causes of the disparate findings related to physiologic variations of cortisol concentration and CV risk, we used three novel approaches: (1) a large sample size, (2) tight environmental control and (3) Framingham risk score as the assessment tool for CV risk. The aim of this study was to determine if there is a positive relationship between physiologic cortisol levels and cardiovascular risk. An additional aim was to determine whether CAV1 risk allele carriers associate with CV risk. Accordingly, we hypothesized that both higher urinary cortisol levels as well as caveolin-1 risk allele carriers would correlate with higher Framingham risk scores; those individuals who had both higher cortisol levels and were CAV1 risk allele carriers would have the highest risk.

Methods

HyperPATH cohort and study protocol

This was a cross-sectional study from the international Hypertensive Pathotype (HyperPATH) cohort. HyperPATH is an ongoing study to investigate the pathophysiologic mechanisms and genes involved in hypertension and CV disease. All subjects completed a common protocol in clinical research centers located at one of five locations: Brigham and Women's Hospital (Boston, MA, USA), University of Utah Medical Center (Salt Lake City, UT, USA), Vanderbilt University Medical Center (Nashville, TN, USA), Hospital Broussais (Paris, France) and San Salvatore Hospital (Rome, Italy). The protocol was approved by institutional review boards of each site, and informed consent was obtained before participant enrollment.

Details of the full protocol have been described previously (25, 26, 27, 28). In brief, subjects were 18–65 years old and had no known or suspected secondary hypertension, coronary artery disease, stroke, renal insufficiency (serum creatinine ≤1.5 mg/dL) or psychiatric illness. All antihypertensive medications were discontinued 3–4 weeks prior to laboratory assessment. Subjects completed the protocol after a 7-day isocaloric high-salt diet (>200 mEq Na/day). On the final evening of the diet week, subjects were admitted to the Clinical Research Center at their respective study site. Blood pressure and laboratory assessments were obtained in the morning between 08:00 h and 10:00 h while fasting and overnight supine posture. Measurement of urine-free cortisol (UFC) was performed on a single 24-h urine collection. UFC was measured by Coat-A-Count Radioimmunoassay (Diagnostics Products Corp., Los Angeles, CA, USA), with a sensitivity of 0.2 μg/dL and a precision of 4–6.4%. Normal reference range for 24-h UFC by radioimmunoassay is considered <100 μg/24 h. For comparison, results of UFC by LC-MS/MS are generally found to be 40% lower than those with radioimmunoassay (29). Details of additional laboratory assays have been described previously (13, 30). Although other results from the HyperPATH cohort have been published, the present analyses are original.

Study end-points

1. *Urinary Free Cortisol:* Prior studies have demonstrated that UFC levels in the population are not normally distributed, but rather bimodally distributed (13, 30). The bimodal distribution is shifted higher in men than women (13, 30). Further, there is evidence of heritability in the higher UFC mode (13, 30). Therefore, subjects in this study were classified as either in the low-mode or high-mode cortisol group based on the bimodal distribution of UFC for their respective sex. As previously described by members of our group, the cut-point to determine low-mode vs high-mode was a UFC value below and above 49 μg/24 h in women and 59.7 μg/24 h in men (30).

2. *Caveolin-1 (CAV1):* We used rs926198 as the candidate variant of CAV1. This single-nucleotide polymorphism was selected based on our group's previous analysis of HapMap variants of the *CAV1* gene in relation to insulin resistance in the HyperPATH dataset. This variant is associated with insulin resistance, hypertension and the metabolic syndrome (21, 22). DNA was genotyped as previously described (21). Individuals were classified as non-risk allele carriers if they were homozygote for the major rs926198 allele and were classified as risk allele carriers if they had one or two alleles for the minor rs926198 allele variant.

3. *Framingham Risk Score:* The hard coronary heart disease (CHD) 10-year risk calculator was used to determine Framingham risk score as outlined in the Adult Treatment Panel III (18). Points are assigned based on an individual's sex, age, total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, systolic blood pressure (SBP), treatment for hypertension and cigarette smoking. The total risk score sums the points assigned to each risk factor to estimate the 10-year risk of myocardial infarction and/or coronary death (18). All participants with diabetes were excluded given that diabetes is considered a CHD risk-equivalent (18).

Statistical analysis

Population characteristics for individuals in the low- and high-mode cortisol groups were compared using a Student's *t*-test. Multivariate regression was used to test the association between urinary cortisol and Framingham risk score. Covariates in the model were selected *a priori* and were treated as fixed effects. Covariates included site of study and factors that have clinical importance for known CV risk factors, race and BMI. As age, sex, SBP and cholesterol are already incorporated into the Framingham risk score, they were not duplicated as covariates. Participant relatedness (i.e. sibling status) was incorporated into the multivariate model. We also compared low-mode and high-mode subjects using *propensity score* analyses (STATA PSMATCH2) to ensure that we had assembled study populations balanced on the baseline covariates of race, site of study and BMI. Statistical significance was indicated by *P*<0.05. All statistical analysis was performed using Stata Statistical Software: Release 15 (StataCorp LLC, College Station, TX, USA).

Results

Baseline characteristics in low- and high-mode cortisol groups

The HyperPATH cohort included a total of 574 non-diabetic individuals who had a 24-h UFC collection and complete data to calculate a Framingham risk score (Table 1). A total of 433 individuals met criteria for the low-mode urinary-free cortisol group and 141 individuals for the high-mode urinary-free cortisol group. Individuals in the high mode were on average 2.4 years older (*P*=0.03) and had a higher mean SBP by 9 mmHg (*P*<0.001), diastolic blood pressure (DBP) by 5 mmHg (*P*<0.001), fasting blood glucose by 4 mg/dL (*P*=0.01), TC by 14 mg/dL (*P*<0.001) and LDL by 14 mg/dL (*P*<0.001) than individuals in the low-mode group.

Framingham risk score higher in high-mode cortisol group

The correlation of individual components of the Framingham risk score with urinary cortisol mode was tested. The low Pearson's coefficient for age (0.10), SBP (0.18), TC (0.15) and HDL (−0.07) indicated the absence of a dominant cardiometabolic risk factor associated with cortisol, further supporting our approach to use the composite Framingham risk score.

On multivariate regression, mean Framingham risk score in the low-mode group was 7.73±0.34 (s.e.m.) vs 10.22±0.43 in the high-mode group (*P*<0.001) (Fig. 1). The propensity score coefficient was 2.39 (95% CI 1.107–3.677), demonstrating adequately balanced groups in regards to the covariates. In the model, higher BMI was independently associated with Framingham risk score. For every increase in BMI of 1 kg/m², Framingham risk score increased by 0.37 points. To provide further validation of our findings, we also assessed the association between urinary-free cortisol as a continuous variable and Framingham risk score. As a continuous variable, higher urinary-free cortisol levels associated with higher Framingham risk score: *P*=0.01.

CAV1 minor allele carriers have higher Framingham risk score

Given the evidence for CV risk heritability and our prior work demonstrating that CAV1 risk allele carriers have greater insulin resistance, we investigated whether

Table 1 Characteristics of study population based on urinary cortisol mode.

	Low-mode (n = 433)	High-mode (n = 141)	β	*P* value
Age (years)	45.3 ± 10.6	47.7 ± 8.6	2.4	0.03
Sex (female, %)	49.7	44.0	−5.7	0.24
Race (white %)	88.7	89.3	0.6	0.84
BMI (kg/m^2)	28 ± 4	28 ± 4	0	0.55
SBP (mmHg)	136 ± 24	145 ± 20	9	<0.001
DBP (mmHg)	81 ± 14	86 ± 12	5	<0.001
UFC (μg/24 h)	34 ± 10	76 ± 25	42	<0.001
Hypertension (%)*	64.1	86.6	22.5	<0.001
Fasting blood glucose (mg/dL)	89 ± 16	93 ± 12	4	0.01
TC (mg/dL)	186 ± 39	200 ± 38	14	<0.001
HDL (mg/dL)	45 ± 19	42 ± 13	−3	0.09
LDL (mg/dL)	114 ± 35	128 ± 36	14	<0.001
TG (mg/dL)	143 ± 101	143 ± 98	0	0.98
Statin use (%)	5.8	8.1	2.3	0.52
Current smoker (%)	10.4	9.0	−1.4	0.62

Values are represented as means ± s.d. for continuous variables and percentages for categorical variables. Comparisons across urinary cortisol modes were performed using Student's *t*-test for continuous variables and Fisher exact for binary variables.

*Hypertension was defined as a seated diastolic blood pressure of ≥100 mmHg off antihypertensive medications or ≥90 mmHg if taking one or more antihypertensive medication.

BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; UFC, urinary-free cortisol.

CAV1 risk allele carriers also had higher Framingham risk scores. Of the 574 individuals who had a 24-h UFC as well as complete data to calculate a Framingham risk score, 517 individuals also had DNA analysis for CAV1 genotype. There were 202 subjects who were CAV1 non-risk allele carriers and 315 subjects who were CAV1 risk allele carriers (247 heterozygotes and 68 homozygotes), and this genotype distribution was in Hardy–Weinberg equilibrium. CAV1 risk allele carriers had a mean±s.e.m. Framingham risk score of 8.91±0.37 compared to CAV1 non-risk allele carriers of 7.59±0.48 (P=0.03) (Fig. 2). As a control, we looked at two other genes in this same population, angiotensinogen and serum/glucose regulated

kinase 1, which are both associated with salt sensitive hypertension (31, 32). We found no relationship between Framingham risk score and angiotensinogen (P=0.22) or serum/glucose regulated kinase 1 risk allele (P=0.75) carrier status.

CAV1, cortisol and Framingham risk score

CAV1 risk allele carriers were no more likely to be in the low-mode vs high-mode cortisol group (P=0.37). However, given the association of urinary cortisol and CAV1 genotype with Framingham risk score, we tested
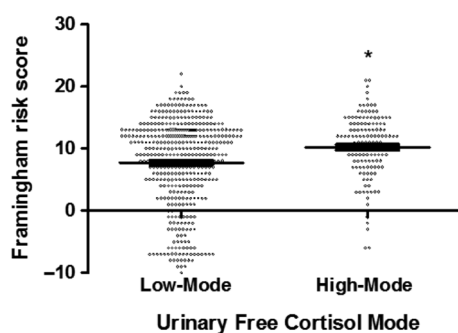
Figure 1
Framingham risk score by low vs high-mode urinary cortisol. Values represent the mean ± standard error of the mean (s.e.m.). Mean Framingham risk score in low-mode was 7.73 ± 0.34 and in high-mode was 10.22 ± 0.43. Multiple linear regression P < 0.001 adjusted for BMI, study site, race and family relatedness.
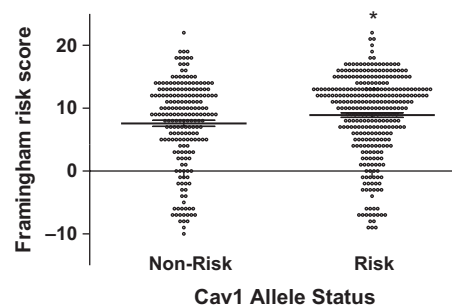
Figure 2
Framingham risk score of Cav1 non-risk vs risk allele (rs926198) carriers. Values represent means ± s.e.m. Mean Framingham risk score in non-risk allele carriers was 7.59 ± 0.48 vs 8.91 ± 0.37 in risk allele carriers. Multiple linear regression P = 0.034 after adjusting for BMI, study site, race and family relatedness.

whether the addition of CAV1 risk allele status as a covariate added meaningfully to our multivariate model of Framingham risk score and urinary-free cortisol mode.

In the low-mode UFC group, the presence of a CAV1 risk allele increased Framingham risk score by 1.2 points (non-risk allele carriers 7.15±0.63; risk allele carriers 8.38±0.47). In the high-mode UFC group, CAV1 risk allele status mediated an approximate 1.0 point increase in Framingham risk score (non-risk allele carriers 9.78±0.47; risk allele carriers 10.77±0.66) (Fig. 3). The inclusion of CAV1 risk allele status contributed significantly to the model. Overall, the estimated effect of the risk allele was an increase in Framingham risk score of 1.33±0.61 (*P*=0.03).

Discussion

Confirming our hypothesis, this study among nearly 600 subjects demonstrated a strong relationship between 24-h UFC levels and predicted CV risk. We have also shown that the CAV1 risk allele was associated with Framingham risk score independently and after incorporation of an individual's 24-h UFC. The lowest Framingham risk scores were those in the low-mode cortisol group and who were CAV1 non-risk allele carriers, while the highest risk scores were those in the high-mode cortisol group and who were CAV1 risk allele carriers, a difference of 3.5 points.

The associations of cortisol and CAV1 on Framingham risk score have meaningful clinical consequences. Even a 1–2 point difference in Framingham risk score can increase a person's 10-year CV risk substantially. For example, men in the low-mode cortisol group had a 10-year CV risk of 4% compared to men in the high-mode cortisol group, whose risk was 6% (18). Those in the high-mode cortisol
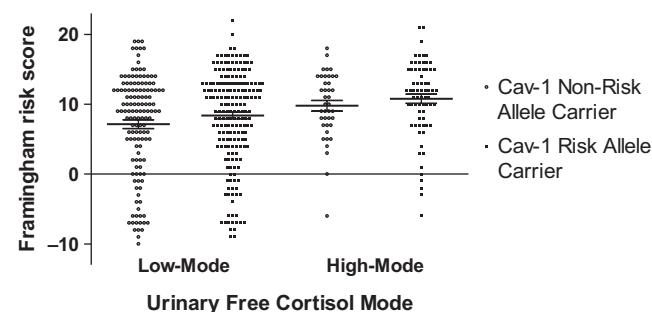
Figure 3
Framingham risk score in Cav-1 non-risk allele vs risk allele carriers grouped by low and high-mode urinary-free cortisol. Values represent means ± s.e.m. Mean Framingham risk score in low-mode/non-risk allele carrier was 7.15 ± 0.63 vs low-mode/risk allele carrier of 8.38 ± 0.47. Mean Framingham risk score in the high-mode/non-risk allele group was 9.78 ± 0.76 vs high-mode/risk allele of 10.77 ± 0.66.

group and CAV1 risk allele carriers had a 2- to 2.5-fold higher 10-year CV risk (8%) compared to those in the low-mode cortisol group and non-risk allele carriers (3%).

Our results provide support that there is a positive relationship between physiologic cortisol levels and CV risk. Over 20 prior studies have investigated whether cortisol measurements are related to components of CV risk. These studies have used various cortisol measurement techniques (morning plasma, salivary, post-dexamethasone suppression, 24-h urine) that assess distinct aspects of the hypothalamic–pituitary–axis. An emerging new technique that may also be of use is measurement of hair cortisol (33, 34). However, often the results from different studies have been variable. For example, in a study among 370 men, morning fasting plasma cortisol concentrations were significantly associated with higher blood pressure, plasma glucose concentrations, fasting triglyceride levels and insulin resistance (11). Conversely, in a study among 369 overweight/obese subjects and 160 healthy volunteers, salivary cortisol, 24-h UFC and post-dexamethasone serum cortisol levels did not strongly relate to obesity and the metabolic syndrome (17). The major difficulty with many of these studies is the substantial variability used to control the factors known to modify the reliability of cortisol measurements, and thereby, obscure the ability to identify a positive association. For example, time of day, posture, sleep–wake cycles, eating and stress can modify circulating cortisol levels over a short time interval. The often variable urine collection times and the difference between cortisol's diurnal and creatinine's non-diurnal cycle can adversely affect using 24-h cortisol excretion rates as an index of cortisol production. Also, the potential confounding effects of medications and diet introduce additional heterogeneity in the measurement of cortisol levels. We believe our study improves upon prior research as we had the largest sample size reported to date. In our cohort, the following controls were used: controlled diet, time of day, daily activity and stress factors in a Clinical Research Center setting. The subjects were not taking any medications. Also, we used 24-h cortisol levels as our outcome measurement. Through these techniques, we minimize the potential 'noise' in assessing cortisol production. Finally, we also are the first to use the highly validated Framingham CV risk score calculator as our method of assessment of CV risk, which incorporates age, TC, HDL, SBP, smoking status and sex and we further corrected for BMI and race.

Our findings related to CAV1 risk allele carriers and increased CV risk are consistent with prior research. Previously, we have shown that the CAV1 risk allele was associated with the metabolic syndrome in both Caucasian

and Hispanic cohorts (21). Building on our prior findings, we have also now demonstrated higher Framingham risk scores for the same CAV1 variant as well as the fact that cortisol and CAV1 are independently associated with CV risk. The mechanism by which cortisol may raise CV risk is not clearly understood. There is evidence implicating a role of glucocorticoids in the modulation of vascular function and reactivity (35, 36). Likewise, the pathophysiology of CAV1 in the development of CVD is unknown, but may be related to a role of CAV1 in mineralocorticoid receptor signaling (28).

These findings have importance in potentially identifying novel CV risk factors and may provide insight into the CVD development. Given the association between physiologic cortisol elevations and CV risk, there may be therapeutic benefit of investigating ways of decreasing cortisol in individuals with greater production. As we move toward personized medicine, the CAV1 risk allele may be more readily available clinically and should alert medical providers of increased CV risk.

Limitations of the study include the cross-sectional design, which prevented us from evaluating a causal relationship between UFC and CV risk. Our findings are highly suggestive but do not prove that physiologically higher cortisol production mediates CV risk. An additional limitation is that we have only one UFC collection for each participant. While our results would be strengthened if we had two UFC collections per participant to account for variability in UFC collections, given that collections were performed under a highly controlled environment, it is anticipated that much of the variability of day-to-day UFC collections would be minimized. We acknowledge that individuals in the high-mode group could have undiagnosed Cushing's or subclinical Cushing's syndrome. However, in the majority of cases, the UFC level was in the high-normal range for the assay. Further, these subjects had no history or clinical evidence of Cushing's syndrome at the time of the study. However, radiographic imaging of the abdomen was not performed to assess for adrenal tumors and neither dexamethasone suppression tests nor late night salivary cortisol were obtained.

Conclusion

In conclusion, with a large sample size and after controlling for environmental factors, we have shown a positive relationship between physiologic, yet higher, urinary cortisol levels and CV risk as well as *CAV1* gene variants and CV risk. As CAV1 risk allele is heritable and high-mode UFC is likely heritable, we add to and further characterize evidence for a heritable component of CV risk.

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