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Sex Differences in the Genetic Basis of Morning Serum Cortisol Levels: Genome-Wide Screen Identifies Two Novel Loci Specific to Women

Lianne M. Kurina, Lauren A. Weiss, Steven W. Graves, Rodney Parry, Gordon H. Williams, Mark Abney, and Carole Ober

Departments of Health Studies (L.M.K.), Human Genetics (L.A.W., M.A., C.O.), and Obstetrics and Gynecology (C.O.), The University of Chicago, Chicago, Illinois 60637; Department of Chemistry and Biochemistry (S.W.G.), Brigham Young University, Provo, Utah 84602; School of Medicine (R.P.), University of South Dakota, Sioux Falls, South Dakota 57105; and Cardiovascular Endocrinology Section (G.H.W.), Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Context: Relatively little is known about the influence of specific genes on cortisol levels, particularly morning cortisol levels.

Objective: The objective of this study was to identify quantitative trait loci associated with morning serum cortisol levels.

Design: We carried out a genome screen for morning serum cortisol using linkage and association methods tailored for use in large pedigrees. We conducted these analyses both in the whole sample and partitioned by sex.

Setting: This study was conducted on nine communal Hutterite farms in South Dakota.

Participants: The Hutterites are a young founder population who practice a communal, farming lifestyle in the western United States and in Canada. Hutterites (n = 504, 53% female) aged 11–89 yr from a single pedigree participated in this study.

Main Outcome Measures: The main outcome measures were markers significantly linked or associated with variation in morning serum cortisol levels.

Results: One genome-wide significant association was identified in the whole sample on 11p (D11S1981, $P = 0.000092$). Results of sex-partitioned analyses indicated that this association was restricted to females (females, $P = 0.000084$; males, $P = 0.20$). The 146-bp allele at this locus accounted for 7% of the variance in morning cortisol values in females, and females homozygous for the allele had an 89% increase in morning cortisol levels compared with female noncarriers. A second genome-wide significant association in females was identified on 14q (D14S74, $P = 0.000091$).

Conclusions: Our results suggest that the genetic determinants of morning cortisol levels may be different for men and women and that loci on 11p and 14q influence morning cortisol levels in women. **(*J Clin Endocrinol Metab* 90: 4747–4752, 2005)**

CORTISOL IS A metabolic hormone that plays a key role in the physiological stress response. Cortisol levels follow a relatively predictable circadian rhythm with an early morning peak after awakening, a rapid decrease over the next few hours, and then a more gradual decline over the course of the day to very low levels by bedtime (1, 2). This rhythm is disrupted by stress, when the sympathetic nervous system prompts a dramatic rise in cortisol levels. Persistently high levels of cortisol have serious health consequences, suppressing immune function and negatively affecting cardiovascular, neural, and gastrointestinal function (3).

Changes in cortisol secretion, either the pattern or total amount, are associated with a number of diseases such as depression (4–6), hypertension (7, 8), rheumatoid arthritis (9), fibromyalgia (10), asthma (11), and Alzheimer's and Parkinson's disease (12). Although it is generally assumed that aberrant cortisol levels are a consequence of disease, there has been speculation that alteration in cortisol secretion might predispose to some of these conditions (13). In support of this hypothesis, Rosmond *et al*. (14) have shown an association between abnormal secretion patterns of both cortisol and testosterone at baseline and an increased 5-yr incidence of cardiovascular disease, type 2 diabetes, and hypertension in a cohort of middle-aged Swedish men. Moreover, cross-sectional studies have identified associations between morning cortisol (defined either as the peak level or as the difference between waking and the peak level) and symptoms of metabolic syndrome, such as visceral obesity, high blood pressure, and a poor lipid profile, although the relationships are complicated and modified by both sex and age (15–18).

Differences in cortisol secretion patterns among individuals could potentially result from prolonged stress (19), which has been associated with disease onset and progression, particularly for infectious disease (20). However, there may also be genetic differences in baseline cortisol secretion that could predispose to, or modify the severity of, various illnesses. Relatively little is known, however, about the influence of specific genes on cortisol levels, particularly morning cortisol levels. Previous studies have shown associations between variants of the genes encoding corticotropin-releas-

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Abbreviations: ASHBD, Allele-specific HBD, CBG, cortisol-binding globulin; GTAM, general two-allele model; HBD, homozygosity by descent; HRT, hormone replacement therapy; QTL, quantitative trait locus; RSS, residual sum of squares.

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ing hormone (*CRH*), on chromosome 8q, and the glucocorticoid receptor (*NR3C1*), on chromosome 5q, and afternoon and evening cortisol levels, but not morning cortisol levels (21, 22). A previous genome-wide linkage scan reported some evidence that an *IGF2 Apa*I polymorphism (11p) was linked to morning serum cortisol in African-American families (LOD > 1.18) but not in Caucasian families in the study (23).

To identify genetic determinants of cortisol secretion, we initiated a study of morning serum cortisol in the Hutterites, a founder population of European descent. Because the Hutterites live on large communal farms and have very similar lifestyles, environmental heterogeneity is minimized, an important advantage for mapping complex traits. Previously, we estimated the narrow heritability of morning serum cortisol to be 0.45 in this population (24), which is consistent with estimates derived from twin and family studies in other European populations (25–27), and indicates that 45% of the variance of this trait is due to additive genetic variation segregating in the population.

Here, we present the results of genome-wide linkage and association screens for quantitative trait loci (QTLs) that influence morning serum cortisol levels in the Hutterites. Because there is evidence of sexual dimorphism in overall plasma cortisol levels (28) and in cortisol response to stressors (29–32), we carried out both unpartitioned and sex-partitioned analyses. We report evidence of two regions that influence morning serum cortisol in females only, suggesting sex-specific regulatory pathways for morning cortisol.

Subjects and Methods

Subjects

The Hutterites are a young founder population who practice a communal, farming lifestyle, which results in a remarkably uniform environment in the population. Details of the population, sampling strategy, and the utility of this population for mapping complex traits have been described previously (24). The 806 Hutterites in our studies are related to each other through multiple lines of descent in a known pedigree. The mean inbreeding coefficient of the individuals in this sample is 0.034 (SD 0.015), slightly greater than that of 1.5 cousins. Morning serum cortisol was measured in 518 adult Hutterites living on nine communal farms in South Dakota after informed consent was obtained, as described elsewhere (24). This study was approved by the Institutional Review Boards of the University of Chicago and the University of South Dakota.

Twelve of the women in this sample who were pregnant at the time of our study were excluded because cortisol levels rise during pregnancy (33), and an additional two individuals (one male, one female) with very low cortisol values identified as outliers by the Grubb test (34) were also excluded, yielding a final sample size of 504. One of the two outliers was taking prednisone; none of the other study participants was taking oral steroids. Six individuals in the sample with asthma reported inhaled steroid use. Exogenous estrogen stimulates increased production of cortisol-binding globulin (CBG), and it has been reported that hormone replacement therapy (HRT) users have higher baseline levels of plasma cortisol (35). In our sample, 23 women reported currently taking HRT (Premarin, Wyeth Pharmaceuticals, Inc., Philadelphia, PA).

Serum collection and cortisol measurement

Individuals donated one morning fasting blood sample within approximately 1 h of waking. Serum samples were allowed to clot for 30 min, centrifuged for 10 min, and then stored and shipped on ice the same day to Boston, where the analyses were performed. Cortisol was measured in the fasting serum samples using the method of Taylor *et al.* (36). The intra- and interassay coefficients of variation for low concentrations of cortisol (3.0 μg/dl) were 4.5 and 6.2%, respectively. The intra- and interassay coefficients of variation decreased to 3.7 and 4.8% for higher cortisol concentrations (30 μg/dl). Cortisol values were normalized with a logarithmic transformation before analysis. To examine the potential influence of age, sex, inhaled steroid use, and HRT use on morning cortisol values, we performed a generalized linear regression of the transformed cortisol values, weighted by the estimated covariance matrix, obtained as previously described (37).

Genotyping

A genome screen using 658 autosomal microsatellite markers (Marshfield screening sets 9 and 51) was completed by the Mammalian Genotyping Service of the National Heart, Lung, and Blood Institute, yielding an approximately 5-cM map (http://research.marshfieldclinic.org/genetics/). In addition, 233 microsatellite markers and 412 intragenic single nucleotide polymorphisms or short insertion/deletion polymorphisms in regions or genes related to asthma and cardiovascular diseases were genotyped in this sample (38, 39). None of these markers was selected because they were functional or positional candidates for cortisol levels. Genotyping was performed blind to all phenotypic information. Distances for framework markers are based on the DeCode map (40) whenever possible; all other markers were placed using the physical map (http://genome.ucsc.edu/) and estimates of recombination within the Hutterite pedigree by CRI-MAP (http://www.hgmp.mrc.ac.uk/Embnetut/Crimap/). The final map had an average intermarker distance of 2.5 cM.

Heritability

The methods used to estimate narrow (h^2) and broad (H^2) heritability using a variance component maximum likelihood method were described in detail elsewhere (41) but will be briefly reviewed here. This method estimates additive, dominance, and environmental variance by using information about the kinship coefficient and the probability of a given pair of individuals sharing two alleles identical by descent without either being autozygous. Accurate estimation of the dominance variance, as opposed to just a sibship correlation, is possible because essentially every pair of Hutterites has a nonzero probability of sharing two alleles identical by descent. We considered models that had, in addition to an environmental variance component, only additive variance, only dominance variance, and both additive and dominance variance components. To assess the best fitting model, we compared the Bayesian information criterion (42) for each model and used the likelihood ratio χ^2 test to determine which components were significant.

Mapping

Genome scans for cortisol QTLs were performed using homozygosity by descent (HBD) linkage and two association methods, as previously described (37, 43). The linkage method tests for correlations between regions inherited HBD and the trait value. The first association method, called allele-specific HBD (ASHBD), is a multipoint method that tests for correlations between specific alleles at markers inherited HBD and the trait value. The second association method is single point and uses a general two-allele model (GTAM). GTAM allows for a quantitative trait to follow any two-allele model (including dominant, recessive, and additive), in contrast to the ASHBD test, which relies on the existence of regions that are HBD in inbred individuals to detect QTLs that act in a recessive manner (37). In the GTAM analyses, the effect of an allele was represented as a main effect in a linear model, as previously described (37). All analyses were first run on the entire sample and then separately by sex. In the sex-specific analyses, the phenotype values for individuals of the opposite sex were entered as missing data.

For all three methods, we assessed empirical significance for each locus using a Monte Carlo permutation test, as previously described (37). Our permutation test preserves the covariance structure due to relatedness among individuals and allows us to assess significance in the presence of multiple, dependent tests and to guard against deviations from normality in the data. Locus-specific *P* values, based on Gaussian theory, were also Bonferroni-corrected to adjust for multiple tests when several alleles were present at a marker locus. The Bonferroni-corrected

P values were very close to the locus-specific permutation-based *P* values (37), so the Bonferroni-corrected *P* values are reported here.

Genome-wide *P* values were further adjusted for the total number of loci tested, and genome-wide significance was inferred when the 95% confidence intervals of these *P* values overlapped with 0.05. Suggestive significance was met if the *P* value at a locus was less than the expected minimum *P* value under the null hypothesis, which was estimated by finding the minimum locus-specific *P* value for each of 1000 permutations and averaging these values.

Although likelihood ratios were not calculated, we can assign an equivalent one degree of freedom LOD score to each of our *P* values using the formula $\text{LOD} = 0.217\text{F}^{-1}(1 - P)$, where F^{-1} is the inverse cumulative distribution function of a χ^2 random variable with one degree of freedom.

Effect size estimation

To estimate effect size for QTLs identified by GTAM, we performed a generalized linear regression of the transformed cortisol values on the covariates, weighted by the estimated covariance matrix, obtained as described in Abney *et al.* (37). We performed this twice for each allele tested, once under the null hypothesis, without genotype data, and once under the alternative hypothesis, with genotype data included as additional covariates. To estimate the percent variance explained by an allele, we calculated the residual sum of squares (RSS) for each regression and used the equation: $(\text{RSS}_{\text{null}} - \text{RSS}_{\text{alt}})/\text{RSS}_{\text{null}}$. We were unable to estimate effect size for QTLs identified by HBD or ASHBD because these methods only test the null hypothesis of no linkage or association at a given locus. As a result, there is no model estimation procedure, as there is in the GTAM.

Results

Characteristics of the study population

The age of the study participants ranged from 11–89 yr; the mean age was 35.4 yr (SD 16.1 yr). Of the participants, 53% were female. All of the participants were Caucasian. Morning serum cortisol levels ranged from 8–47.5 μg/dl, with a mean of 20.6 μg/dl (SD 5.9). These values are similar to peak morning values reported for other healthy populations, although the Hutterite mean is slightly higher (12, 28, 44, 45). Morning cortisol levels were not predicted by age ($P > 0.05$) but were slightly higher in women than in men ($P = 0.06$). Morning cortisol levels were not influenced by inhaled steroid use ($P > 0.05$) or by HRT use among women over 50 yr of age ($P > 0.05$).

Estimates of heritability

Estimates of the broad and narrow heritabilities were similar in all of the samples tested. In the pooled sample, the broad and narrow heritabilities were 0.54 (SE 0.09), indicating that 54% of the variance of this trait is due to genetic variation segregating in the population. This value differed slightly from that previously reported in the Hutterites because here we excluded 12 pregnant women and two individuals with outlier values (see *Subjects and Methods*). The broad and narrow heritabilities were 0.52 (SE 0.14) in the female-only analysis and 0.53 (SE 0.14) in the male-only analysis. In all cases, the additive model provided a better fit to the data than models including dominance components or only environmental components.

HBD linkage analysis

No linkage signals met criteria for suggestive or genome-wide significance in the pooled sample. Two regions in the female-only analysis and one region in the male-only analysis met the criterion for suggestive significance (locus $P < 0.002$) (Fig. 1). In the female-only analysis, linkage peaks were detected on 5q at 169 cM (LOD = 2.3, locus $P = 0.0012$) and on 10q at 97 cM (LOD = 2.7, locus $P = 0.00046$). In the male-only analysis, a linkage peak was detected on 4p at 7 cM (LOD = 2.1, locus $P = 0.0016$).

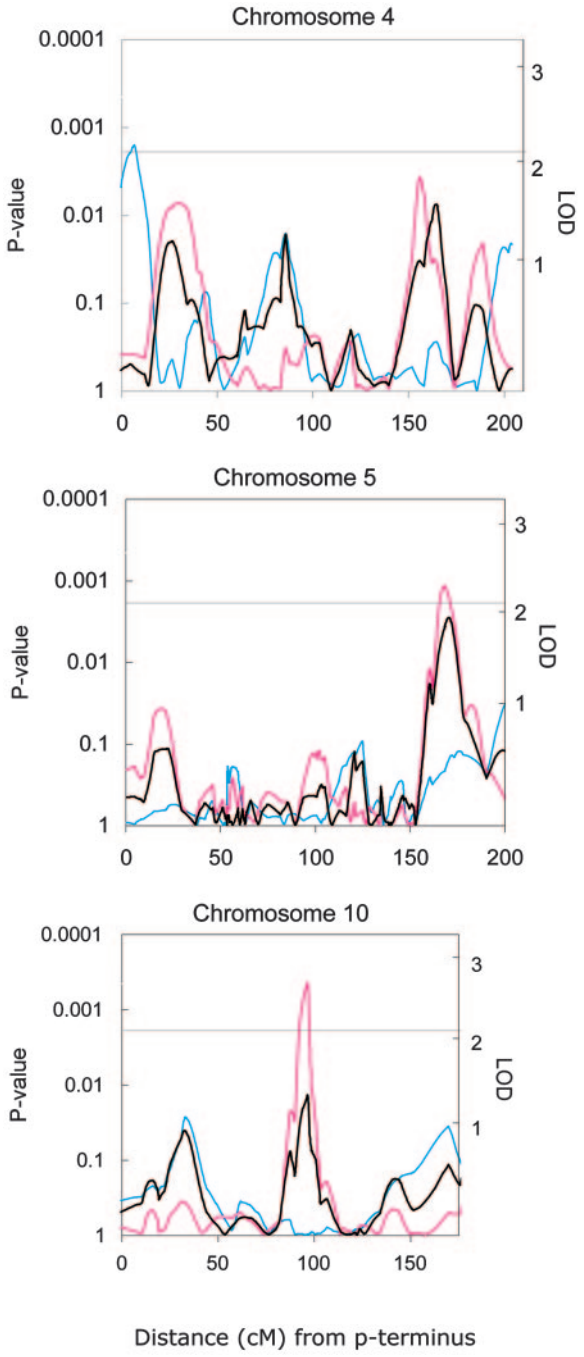

FIG. 1. Results of HBD linkage mapping for serum morning cortisol that met criteria for suggestive significance. Locus *P* values are shown on the *left axis* and LOD scores on the *right axis*; the *gray line* denotes the threshold for suggestive significance (see *Subjects and Methods*). Results in the full sample are shown as a *black line*, in males only as a *blue line*, and in females only as a *pink line*.

ASHBD association test

No associations met criteria for suggestive or genome-wide significance in the pooled sample or in the male-only sample in the ASHBD analysis. In females, one association on chromosome 14q at 78 cM (D14S74) met the criterion for genome-wide significance, with a locus $P = 0.000091$ (Table 1).

GTAM association test

One association, on chromosome 11p at 26 cM (D11S1981), met the criterion for genome-wide significance in both the pooled sample (locus $P = 0.000092$) and female-only analyses (locus $P = 0.000084$) (Table 1). One association on chromosome 6p at 31 cM (D6S2434) met the criterion for suggestive significance in the pooled sample, with a locus $P = 0.00013$. No associations were revealed in the male-only analysis.

Effect size estimates

Effect sizes were estimated for D11S1981 in the female-only and pooled samples. The associated 146-bp allele accounted for 7% of the variance in morning cortisol in females and 4% of the variance in the pooled sample model. At this locus, the effects appear additive; female heterozygotes had a 38% increase in morning cortisol levels (multiplicative effect size = 1.38), and female homozygotes had an 89% increase in morning cortisol (multiplicative effect size = 1.89) compared with noncarriers. In the pooled sample, heterozygotes had a 27% increase in morning cortisol (multiplicative effect size = 1.27), and homozygotes had a 62% increase in cortisol (multiplicative effect size = 1.62), compared with noncarriers. The larger effects in females compared with the pooled sample, both in terms of percent variance explained and effect size, together with the sex-specific GTAM results suggest that the 11p locus is more important in females than in males.

Discussion

This is the first report of genome-wide significant associations for morning serum cortisol. In addition to identifying two novel loci related to morning cortisol, our study provides evidence of the important influence of sex on the genetic architecture of this trait. Although we observed an association for morning cortisol in the pooled sample on chromosome 11, results of the sex-partitioned analyses and effect size estimates indicated that the association on chromosome 11 was specific to women. A second association with morning cortisol in women was observed on chromosome 14. Finally, female-specific linkage peaks reaching suggestive significance were detected on chromosomes 5 and 10, whereas a male-specific linkage peak reaching suggestive significance was detected on chromosome 4. These data suggest that there are different genes involved in the regulation of morning cortisol in men and women. Of note is that no signals were observed near the loci encoding the genes for corticotropin-releasing hormone (*CRH*) on chromosome 8q or the glucocorticoid receptor (*NR3C1*) on chromosome 5q. This may not be surprising given that previous studies showed associations between variants of these genes and afternoon and evening cortisol levels but not morning cortisol levels (21, 22).

Cortisol levels rise quickly in the morning, reaching a peak at about 30 min after waking and dropping quickly thereafter, a pattern known as the cortisol awakening response. In our study, fasting blood samples were collected within 1 h of awakening. Therefore, it is likely that the QTLs identified in our study are markers for genes that contribute to the regulation of this awakening response. These genes could be influencing the cortisol awakening response by, for example, changing its timing relative to waking or by affecting the negative feedback process integral to maintaining cortisol levels within certain bounds (46, 47). Although it is possible that the anticipation of a blood draw could have induced a stress response in some individuals, thereby raising their cortisol levels, the fact that the heritability estimate in the Hutterites (54%) is relatively high and similar to estimates in other populations (one of which was based on salivary cortisol levels, which would avoid the stressor problem) (25–27), suggests that this is not likely. Another possible source of variation in the morning serum cortisol values is the presence of a medical condition associated with changes in cortisol secretion patterns such as major depression (48). The effects of stress or disease would presumably add noise to the cortisol measurements and reduce the power of the study to detect regions associated with morning cortisol. The fact that we observed two genome-wide significant associations in women indicates that genetic influences on morning cortisol were sufficiently strong to be detected, despite the possible sources of phenotypic variability discussed.

Finally, it is important to note that in this study we measured total serum cortisol, not free cortisol. Sex differences in levels of CBG could, therefore, potentially affect our results (49, 50). However, we excluded pregnant women from the

TABLE 1. Genome-wide (GW) significant associations with morning serum cortisol in the Hutterites

	Associated marker	
	D11S1981	D14S74
Association test	GTAM	ASHBD
Chromosome (distance from p-ter)	11p (26 cM)	14q (78 cM)
Associated allele (frequency)	146 bp (0.037)	301 bp (0.275)
Model (direction of effect)	Additive (+)	HBD (+)
Locus P_{all} (GW P_{all}; 95% CI)	9.2×10^{-5} (0.055; 0.040, 0.069)	0.02 (1.0)
Locus P_{female} (GW P_{female}; 95% CI)	8.4×10^{-5} (0.059; 0.044, 0.074)	9.06×10^{-5} (0.062; 0.047, 0.077)
Locus P_{male} (GW P_{male})	0.20 (1.0)	0.49 (1.0)
Positional candidate gene (distance from associated marker)	*ABCC8*, sulfonylurea receptor (300 kb)	*SKIIP*, transcriptional coactivator for vitamin D receptor and retinoid receptors (300 kb)

See *Subjects and Methods* for description of analytical methods and assessment of significance. CI, Confidence interval.

analysis, eliminating one of the major sources of variation in female CBG levels. Furthermore, if CBG was a major factor influencing the associations, then one would expect an age association in serum cortisol levels for women but not for men, and we did not observe any such effect (data not shown).

Females homozygous for the 146-bp allele at D11S1981 on chromosome 11 had nearly double the level of morning cortisol compared with noncarriers, whereas in the pooled sample, homozygosity was associated with a 62% increase in morning cortisol levels. A previous genome-wide linkage scan reported some evidence that an *IGF2 Apa*I polymorphism (11p15.5; 0.7 Mb) was linked to morning serum cortisol in African-American families (LOD $>$ 1.18) but not in the Caucasian families in the study (23). The linkage peak was approximately 20 cM distal to D11S1981, where the association was detected in the Hutterites. None of the markers closer to *IGF2* were associated with morning cortisol in the Hutterites, making it unlikely that we are detecting the same signal. On the other hand, D11S1981 is located approximately 300 Kb from the *ABCC8* gene, which encodes the sulfonylurea receptor. This receptor and the inwardly rectifying potassium channel together make up the ATP-sensitive potassium channels that control insulin secretion in pancreatic β cells. *ABCC8* is an interesting candidate gene for morning cortisol in light of the key role cortisol plays in metabolic processes and the substantial current interest in the relationship between cortisol abnormalities and development of obesity and metabolic syndrome (15–18, 51, 52). Although a recent large case control study and meta-analysis of type 2 diabetes did not find an association with *ABCC8* (53), an association has been reported between *ABCC8* and a prediabetic trait named the disposition index, which describes β-cell compensation for insulin resistance (54). Therefore, we consider the *ABCC8* gene a viable candidate for the morning cortisol locus on 11p in Hutterite females.

The second female-specific association that we identified on 14q with D14S74 is located approximately 300 Kb from *SKIIP*, which encodes the ski-interacting protein, a transcriptional coactivator that binds to the ligand-binding domain of the vitamin D and retinoid receptors to enhance vitamin D-, retinoic acid-, estrogen-, and glucocorticoid-mediated gene expression (55, 56). *SKIIP* is an interesting candidate for the 14q locus associated with morning cortisol in Hutterite women because it mediates cortisol-induced gene expression.

The sex-specific genetic architecture of morning cortisol revealed by our data adds to a growing body of literature showing sex specificity of loci linked or associated with other traits such as glucose tolerance (57), serotonin levels (43), inflammatory bowel disease (58), osteoarthritis (59), and metabolic syndrome (60). Interestingly, in this latter study, most of the markers that were significantly associated with metabolic syndrome were detected in females only, similar to our study of morning cortisol levels in the Hutterites.

The success of this genome scan in identifying regions that likely contain QTLs for morning serum cortisol may be due in part to the reduced environmental variation among individuals in the study population. Cortisol secretion is affected by a number of environmental/lifestyle variables such as diet, physical exercise, and sleep schedule (30, 61, 62). The Hutterites' communal lifestyle ensures that all members of this community have very similar diets, activities, and schedules, and this reduction in environmental heterogeneity likely enhanced our ability to detect genetic influences on this trait. The regulation of cortisol secretion is complex, and the identification of two novel loci should help direct future research efforts to expand our understanding of the genetic determinants of cortisol secretion. In conclusion, this is the first report of genome-wide significant associations with morning serum cortisol and the first suggestion that the genetic underpinnings of cortisol secretion may vary for men and women.

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Address all correspondence and requests for reprints to: Lianne Kurina, Department of Health Studies, The University of Chicago, 5841 South Maryland Avenue, MC2007, Chicago, Illinois 60637. E-mail: lkurina@uchicago.edu.

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