



Chapter V.B

The Low Single Nucleotide Polymorphism Heritability of Plasma and Saliva Cortisol Lev- els

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ABSTRACT

Cortisol is an important stress hormone affected by a variety of biological and environmental factors, such as the circadian rhythm, exercise and psychological stress. Cortisol is mostly measured using blood or saliva samples. A number of genetic variants have been found to contribute to cortisol levels with these methods. While the effects of several specific single genetic variants is known, the joint genome-wide contribution to cortisol levels is unclear. Our aim was to estimate the amount of cortisol variance explained by common single nucleotide polymorphisms, i.e. the SNP heritability, using a variety of cortisol measures, cohorts and analysis approaches. We analyzed morning plasma (n=5,705) and saliva levels (n=1,717), as well as diurnal saliva levels (n=1,541), in the Rotterdam Study using genomic restricted maximum likelihood estimation. Additionally, linkage disequilibrium score regression was fitted on the results of genome-wide association studies (GWAS) performed by the CORNET consortium on morning plasma cortisol (n=12,597) and saliva cortisol (n=7,703). No significant SNP heritability was detected for any cortisol measure, sample or analysis approach. Point estimates ranged from 0% to 9%. Morning plasma cortisol in the CORNET cohorts, the sample with the most power, had a 6% [95%CI: 0-13%] SNP heritability. The results consistently suggest a low SNP heritability of these acute and short-term measures of cortisol. The low SNP heritability may reflect the substantial environmental and, in particular, situational component of these cortisol measures. Future GWAS will require very large sample sizes. Alternatively, more long-term cortisol measures such as hair cortisol samples are needed to discover further genetic pathways regulating cortisol concentrations.

INTRODUCTION

Cortisol secretion is regulated by the hypothalamic-pituitary-adrenal axis in response to various biological and environmental factors, including physical stressors such as intensive resistance exercise¹ or injury,² and psychological stressors such as public speaking and demanding cognitive tasks.³ Cortisol secretion has a marked circadian rhythm: secretion peaks shortly after awakening and then drops throughout the day, reflecting the hormone's role in regulating energy metabolism.⁴ Additionally, cortisol is secreted rhythmically resulting in a pulsatile ultradian rhythm.⁵ The combination of these factors leads to substantial systematic and unsystematic variation of cortisol levels throughout the day.

Cortisol levels can be assessed with a variety of methods, the most common being blood in plasma and saliva samples. Plasma samples represent bound and unbound cortisol concentrations, whereas saliva represents the bioactive free cortisol. These measures have a modest to good correlation^{6,7} and have been associated with various traits and states: BMI,⁸ cardiovascular risk factors including hyperglycaemia,⁹ psychiatric disorders, such as post-traumatic stress disorder, schizophrenia or bipolar disorder^{10,11} and treatment response to depression.¹² Saliva cortisol can be sampled non-invasively, which may reduce the chance of inducing stress, makes repeated measurements more feasible, and facilitates mapping of day-time profiles. Repeated cortisol measures tend to show higher between-visit reliability than single measures at awakening or 8am.^{13,14}

Plasma and saliva cortisol have been investigated in twin studies to determine the extent of the genetic contribution underlying the hormone. For acute plasma cortisol measures, the estimates range from low (14%) to moderate heritability (45%).^{15–17} Wüst et al.¹⁸ reported 0% heritability for acute saliva levels at 8am and total day-time profiles, and observed a large contribution of shared environment (>40%). These family studies rely on relatedness information obtained from known familial relationships instead of direct molecular measurements such as SNP arrays. Molecular genetic studies that can clarify the nature and extent of the genetic effects underlying cortisol are lacking, although they could advance our understanding of the genetic contribution to stress vulnerability as assessed by cortisol. A genome-wide association study (GWAS) by the cortisol network consortium (CORNET) successfully detected and replicated one genetic locus associated with morning plasma cortisol levels, suggesting that common autosomal gene variants are associated with this phenotype.¹⁹ It is plausible that a substantial number of variants associated with cortisol were not identified due to stringent multiple testing corrections required in GWAS. If this is the case, then the joint effect of all SNPs should be larger than the variance explained by the locus found (<1%).

In the present study, we aimed to quantify the SNP heritability of cortisol, i.e the variance jointly explained by common autosomal single nucleotide polymorphisms. The SNP heritability information represents a more direct measure of the genetic pre-

disposition to high or low cortisol stemming from additive genetic effects of common gene variants compared to the broad-sense heritability estimated in family studies. SNP heritability can therefore inform future GWA studies about sample size and potential success. We focus on cortisol measured in plasma and saliva measured in elderly participants from the Rotterdam Study and in mixed ages from the CORNET cohorts. This allowed the study of acute morning levels (plasma and saliva) and day-time profiles (saliva) in large sample sizes. SNP heritability can be estimated with different methods. In this study we used genomic restricted maximum likelihood estimation (GREML)²⁰ in the Rotterdam Study as well as LD score regression in the CORNET GWAS results.

METHODS

Rotterdam Study

Participants

The Rotterdam Study is a population-based cohort investigating chronic disease and their risk factors in elderly, see Hofman et al.²¹ for details. The Rotterdam Study includes 14,926 participants aged 45 and older. Study protocols were approved by the medical ethics committee according to the Population Study Act Rotterdam Study, executed by the Ministry of Health, Welfare and Sports of the Netherlands. Written informed consent was obtained from all participants.

Plasma cortisol information was available in 9836 participants performed in 1997-2008. For 8501, complete information on genetics was available. 2796 participants were removed from GREML analyses due to excessive relatedness (see 2.1.2), resulting in a GREML sample of 5705. In the time adjusted analyses, a further 83 were excluded due to missing information regarding timing of sampling.

Saliva cortisol was available in 2034 participants of which 1982 had complete data on genetics. After removal of 265 participants due to excessive relatedness 1717 individuals remained with acute saliva level upon awakening. Of those, 1541 had also information on later time points for total day-time cortisol computations. See Table 1 for participant characteristics.

Measurements

Plasma cortisol was collected from 8:00h to 20:00h. 75% of samples were collected before 10:30 and 99% before 15:30. Cortisol was measured using the LC-MS/MS method with the CHS MSMS Steroids Kit (Perkin Elmer, Turku, Finland) containing 2H3-cortisol as internal standard. Chromatographic separation was performed on a Waters

(Milford, MA, USA) Acquity UPLC HSS T3 1.8 μ m column and quantified by tandem mass spectrometry using a Xevo TQ-S system (Waters, Milford, MA).

Sarstedt Cortisol Salivette collection tubes (Sarstedt, Rommelsdorf, Germany) were used to collect saliva after awakening, 30 min after awakening, at 17:00 and at bedtime by the participants.²² Participants were instructed to note the exact time of saliva collection, and not to eat or brush teeth 15min before collection. An enzyme immunoassay (IBL International GmbH Hamburg, Hamburg, Germany) was used to analyze the samples. We investigated awakening cortisol levels and diurnal cortisol, calculated by the area under the curve in respect to ground (AUCg).

In the Rotterdam Study genotyping was performed using Illumina HumanHap 550v3 and Illumina HumanHap 610. The genotyped dataset was restricted to persons who reported that they were from European descent. Ethnic outliers were further excluded by removing samples which showed more than 4SD difference to the study population mean on any of the first 4 dimensions of a multidimensional scaling analysis. We also excluded samples with gender mismatch and excess autosomal heterozygosity as well as duplicates and monozygotic twins (>97% estimated identity-by-descent proportion). Furthermore, second degree cousins or closer relatives were excluded during the GREML analysis by using a GRM cutoff of 0.025 to avoid bias from shared environment. MACH 1.0 software was used to impute to ~30M SNPs based on the 1000 genomes Phase I version 3 reference panel.²³ SNPs included in imputation met the thresholds minor allele frequency $\geq 1\%$, Hardy-Weinberg equilibrium $p > 10^{-6}$, and a SNP call rate $\geq 98.0\%$.

GREML

SNP heritability of the cortisol measurements in the Rotterdam Study were estimated using individual level data with GREML, as implemented in Genome-wide Complex Trait Analysis (GCTA) 1.25.3.²⁰ GREML quantifies how well the similarity in the genotype between study participants explains the similarity in phenotype. Genetic similarity was established by computing a genetic relatedness matrix (GRM). We used 8,131,668 imputed autosomal SNPs to create the GRM, after filtering for imputation quality ($R^2 > 0.5$) and minor allele frequency (MAF) ≥ 0.01 . The GRM was specified as a random effect predicting cortisol levels. To test whether this genetic effect statistically significantly predicts the phenotype, we compared the GRM to a simpler model without the GRM using a likelihood ratio test.

Visual examinations of the total genetic effect and residuals using QQ-plots showed deviations from normality for the saliva measurements. The distribution was normal after square root transformation of hormone levels for saliva cortisol. A constant (+1) was added before transformation to avoid zero values. We report results from analyses on transformed saliva and untransformed plasma levels. Additionally, we performed a power analysis as described by Visscher et al.²⁴ The plasma cortisol GREML analyses were well powered to detect 16% heritability (power=80% at $\alpha=0.05$ and 2×10^{-5} genetic

relationship). The power to detect SNP heritability was less in the saliva GREML analyses and thus these analyses have less precision.

Covariates and Confounders

We adjusted the phenotype in all analyses for age, sex and four principal components (PC) of ancestry (computed with GCTA). This was achieved by regressing the phenotype on the covariates and using the residuals as outcome in the GREML analysis. The residuals were computed in R 3.2.3.²⁵ Since plasma cortisol levels were measured in three different Rotterdam Study cohorts, a random intercept on the cohort level was introduced in the regression model of plasma cortisol using the lme4 1.1-10 package.²⁶

Additionally, we performed a sensitivity analysis with the plasma data aimed at reducing the environmental variance. This model was adjusted for time and fitted in participants with blood sampling before 11am and no self-reported corticosteroid use (n=4,696). To account for non-linear effects, time-of-day was specified using cubic splines with three degrees of freedom. The residuals, representing time-adjusted plasma levels, were then used in further GREML analyses.

CORNET Consortium Plasma and Saliva Cortisol GWAS

Detailed description of the CORNET GWAS on plasma cortisol can be found in Bolton et al.¹⁹ Briefly, basal morning plasma cortisol was measured in 12,597 participants in 11 western European cohorts. Blood samples were collected between 7am and 11am and analyzed using immunoassays. All participants were at least 17 years old and of European ancestry, were not using glucocorticoids, pregnant, or breast feeding. In total 2945 participants (23%) were included from the Rotterdam Study. However, the measurements were collected in a different study wave than the one used for GREML analyses. HapMap-imputed autosomal SNPs were associated with z-scores of log-transformed plasma cortisol levels in an age, sex and time adjusted additive model. The SNP effects were meta-analyzed with a fixed effect model using inverse-variance weighting. After quality control, the data featured 2,660,191 SNPs with minor allele frequency >2%.

In parallel, an additional GWAS of morning saliva levels was performed. This study is unpublished and therefore is presented in more detail. Morning (at awakening) saliva cortisol was measured in 7,703 participants in 8 cohorts: the British 1958 Birth Cohort-Type 1 Diabetes Genetics Consortium (N=1762); the British 1958 Birth Cohort-Wellcome Trust Case-Control Consortium (N=1052);²⁷ the Netherlands Study of Depression and Anxiety (N=1220);²⁸ the Netherlands Twin Register (N=162);²⁹ the Rotterdam Study I (N=1767); the Rotterdam Study III (N=1119); the Multi-Ethnic Study of Atherosclerosis (N=166);³⁰ and the Tracking Adolescents' Individual Lives Survey (N=455).³¹ Only awakening samples collected before 11 am were included in the analyses. Participants using systemic corticosteroids and pregnant and breast-feeding women were excluded from the analyses. All subjects were at least 16 years old and of European ancestry. Details of the genotyping and imputation are given in Table S2. Genotype quality control was per-

formed in each study separately (HWE P-value $>10^{-6}$, MAF >0.01 , SNP-call-rate $>95\%$). A z-score was calculated (cortisol at awakening per SD-score in the cohort) to standardize cortisol measurements across cohorts. A linear regression analysis was performed on z-scores of morning saliva cortisol levels adjusted for sex, age and genetic ancestry (cohort specific) using all imputed SNPs.

The meta-analysis was performed with a fixed-effects inverse variance model using the software METAL.³² In addition to study-specific pre-imputation quality control, SNPs with a MAF <0.05 and an observed to expected variance ratio (imputation quality) less than 0.3 were excluded at the meta-analysis level. Furthermore, only SNPs with information from 4 or more studies were included, resulting in a final SNP number of 2,156,702 SNPs. Genomic control correction was applied to each study. This GWA morning cortisol saliva meta-analysis has an overlap with the GREML analysis of 1767 participants/measurements (23%) from the Rotterdam Study. QQ and Manhattan plots were created with qqman 0.1.4.³³

LD Score Regression

LD Score regression exploits the relationship between SNP-Phenotype association strengths and linkage disequilibrium (LD) patterns.³⁴ Some SNPs show stronger associations than expected due to chance. Assuming true causal effects, the SNPs which are in higher linkage disequilibrium (LD) with nearby SNPs are expected to have more inflated test statistics, because they are more likely to tag causal variants with stronger effects. This makes it possible to use a LD score of a SNP, defined as the sum of r^2 in a 1cM region, as a predictor of the association strength in a regression. The variance explained by the LD score is equivalent to the SNP heritability estimated by GREML. The advantage of LD score regression is, that it can be conducted with summary data from a GWAS and no individual level information is required. However, this analysis tends to have larger standard errors compared to GREML, which uses individual level data and thus can test SNP heritability effects directly.

The SNP h^2 was estimated using LD score regression 1.0.0³⁴ in the CORNET GWAS data. Since imputation quality can confound LD score regression results, we restricted the analysis to a list of well-imputed SNPs, as recommended by the software

Table 1: Descriptive statistics of the Rotterdam Study cortisol measurements and participant characteristics

Cortisol Phenotype	Median Levels in nmol/l (25%; 75% quantile)	Median Age in years (25%; 75% quantile)	Sex (% female)	Median time of collection in Hr (25%; 75% quantile)
Plasma	345.6 (281.7;418.1)	63.6 (58.2;72.44)	57%	0942 (0900;1030)
Saliva (awakening)	13.15 (8.7;18.8)	74.3 (70.5;78.9)	56%	0730 (0700;0806)
Saliva (AUCg)	7.90 (5.7;10.4)	74.3 (70.5;78.8)	55%	-

authors. After applying default quality control settings (see Table S3), the final SNP number was 1,028,327 for plasma cortisol and 951,308 for saliva cortisol.

RESULTS

SNP Heritability

Descriptive statistics of the plasma and saliva cortisol levels can be found in Table 1. SNP heritability estimates were low for all cortisol measurement methods, analytical approaches, and cohorts. See Table 2 for full results.

Plasma Cortisol

We estimated the SNP heritability of plasma cortisol using individual level data of the Rotterdam Study (n=5,705) with GREML. In this cohort approximately 1% [95%CI: 0-12%] of variance in plasma cortisol could be explained by common autosomal gene variants. Adjusting for time of day and excluding participants with plasma cortisol measurements after 11am or those using corticosteroids did not meaningfully change results.

We further investigated the SNP heritability of plasma cortisol in a larger consortium sample: the CORNET cohorts (ncohorts= 11, nparticipants=12,597). We applied LD score regression to estimate SNP heritability of plasma cortisol across multiple cohorts using

Table 2: SNP Heritability estimates of plasma and saliva cortisol measurements.

Cortisol Phenotype	Analysis Method	Number of SNPs	n	SNP h^2	SE	p
<i>Main Analyses:</i>						
Plasma	GREML	8,131,668	5,705	0.006	0.059	0.460
Plasma	LD Score	1,028,327	12,597	0.061	0.035	-
Saliva	GREML	8,131,668	1,717	0.090	0.200	0.329
Saliva (AUCg)	GREML	8,131,668	1,541	0.041	0.210	0.420
Saliva	LD Score	951,308	7,703	-0.083	0.060	-
<i>Sensitivity Analysis:</i>						
Plasma-11am	GREML	8,131,668	4,696	0.000	0.073	0.500

Analyses were adjusted for age, sex and ancestry. Plasma cortisol GREML analyses were further adjusted for cohort effects. Additionally, a sensitivity analysis with adjustment for time-of-day and a subset of participants with measurements before 11am and no reported corticosteroid use is reported (Plasma-11am). Negative heritability values can occur for LD score regression analyses due to sampling variance.

the summary results of a GWAS meta-analysis. The variance explained for this larger sample was also low with 6% [95%CI: 0-13%].

Saliva Cortisol

In addition to plasma cortisol, we estimated the SNP heritability of two saliva cortisol phenotypes: awakening and diurnal levels. First, we estimated the variance explained of saliva awakening levels in the Rotterdam Study with GREML ($n=1,717$). The heritability in this sample was 9% [95%CI: 0-48%]. Repeating the analysis in the larger CORNET sample ($n_{cohorts}=8$, $n_{participants}=7,703$) using LD score regression on GWAS meta-analysis summary statistics showed a negative heritability estimate (-0.0833). Phenotypes with low heritability can be estimated as negative due to sampling variance, which suggests population heritability close to 0 and an upper 95% confidence interval of 3%. Finally, we estimated the SNP heritability of diurnal cortisol levels (AUCg). These were only

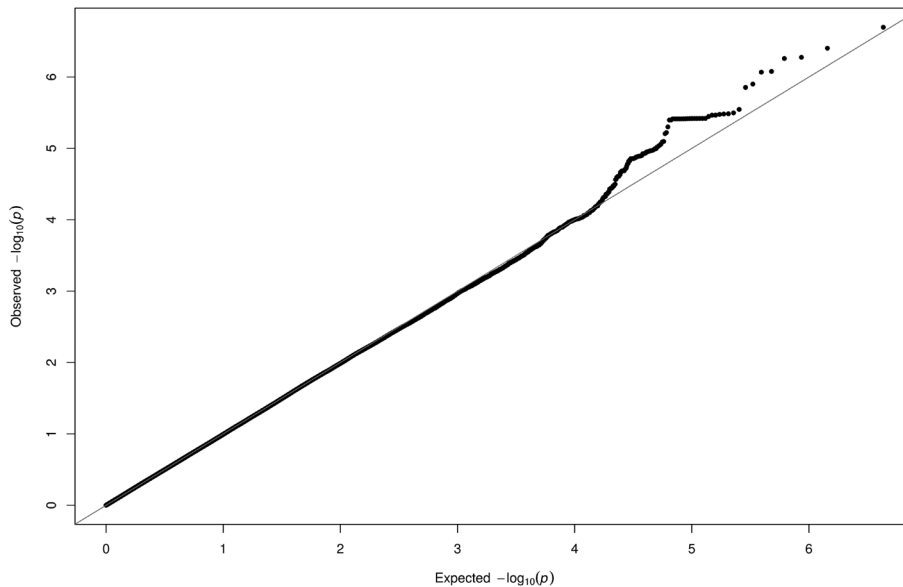


Figure 1: Quantile-quantile plot of observed $-\log_{10} p$ values vs expected $-\log_{10} p$ values assuming chance findings. Diagonal line indicates a p value distribution compatible with chance finding. Upward deviations indicate p values more significant than expected.

available in the Rotterdam Study ($n=1,541$). In this sample the heritability was estimated at 4% [95%CI: 0-45%].

Morning Plasma and Saliva Cortisol GWAS

The CORNET GWAS meta-analysis of plasma cortisol, which was previously published,¹⁹ identified 4 SNPs in the SERPINA6/SERPINA1 locus, namely rs12589136, rs2749527, rs2749529 and rs11621961.

However, no SNP reached genome-wide significance ($p < 5 \times 10^{-8}$) in the GWAS for awakening saliva cortisol. Figure 2 displays a Manhattan plot. Two loci showed suggestive associations ($p < 5 \times 10^{-7}$). The T allele of rs1170109 (chr13:42779694) was associated with a 0.12 SD increase in cortisol levels ($SE=0.02$, $p=3.95 \times 10^{-7}$, $MAF=12\%$, $n=7,690$) with a homogeneous effect across the cohorts ($I^2=0\%$). Several SNPs from the same locus, close to the gene DGKH, showed suggestive effects as well (see Figure 3 for a LocusZoom plot³⁵). The locus was not associated with plasma cortisol ($\beta=0.03$, $SE=0.02$, $p=0.17$, $I^2=0\%$, $n=12,592$). In the second locus, the A allele of rs6768297 (chr3:168334386) was associated with 0.34 standard deviations (SD) lower cortisol levels ($SE=0.06$, $p=2.01 \times 10^{-8}$).

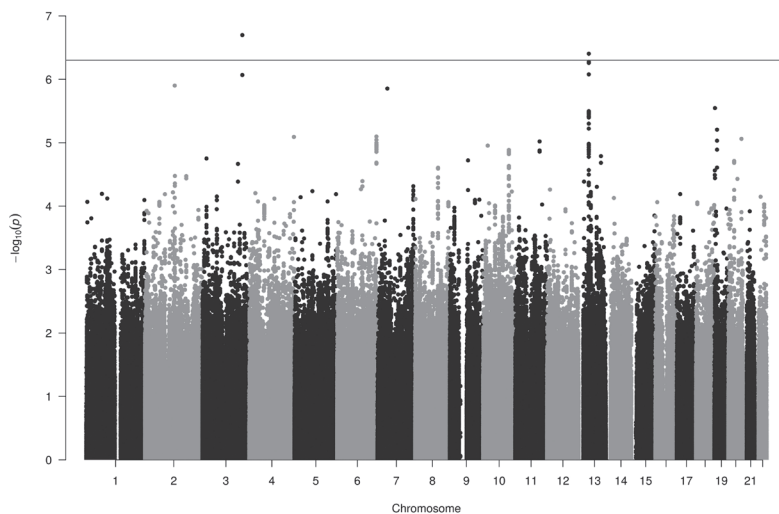


Figure 2: Manhattan plot of $-\log_{10} p$ values vs SNP position. SNPs above the horizontal line indicate suggestive findings ($p < 5 \times 10^{-7}$).

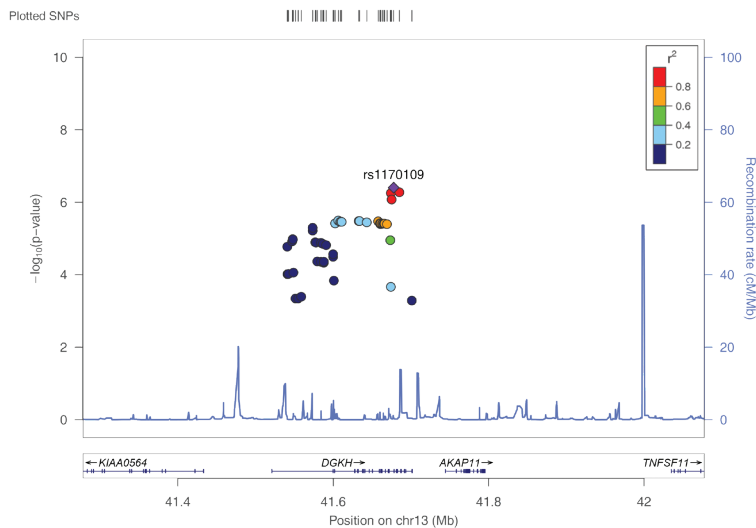


Figure 3: Regional plot around lead SNP rs1170109. $-\log_{10} p$ values of rs1170109 and other top1000 SNPs in the region are displayed color coded for strength of correlation.

7). Furthermore, the SNP showed a nominally significant ($\alpha=0.05$) association with plasma cortisol in the same direction ($\beta=-0.08$, $SE=0.03$, $p=0.01$, $I^2=0\%$, $n=11,441$). Rs6768297 had a low MAF (6%), high effect heterogeneity ($I^2=85.5\%$) and information was only available in 40% of the sample ($n=3054$). None of the four SNPs associated with plasma cortisol were associated with saliva cortisol (all $p<0.56$).

The LD score intercept was 1.0031 ($SE=0.0066$) and 1.0085 ($SE=0.0073$) for the plasma and saliva GWAS, respectively, suggesting no inflation due to population stratification. The QQ plots also showed no problematic inflation (see Figure 1 for saliva).

DISCUSSION

The low heritability of plasma cortisol in two large samples estimated by two different approaches strongly suggests that plasma cortisol is not substantially affected by the additive effects of autosomal SNPs. The same conclusion can be drawn for morning saliva cortisol, which was also estimated by two analytical approaches, and to a lesser extent for diurnal cortisol.

No SNP reached genome-wide significance in a GWAS of morning saliva cortisol levels, which is expected for traits with low SNP heritability analyzed in relatively small samples. Two loci showed suggestive associations. Interestingly, one top SNP rs6768297 lies within the EGFEM1P gene, which has a high and specific expression in the pituitary according to RNA expression data (1.5 reads per kilobase per million).^{36,37} Furthermore, the SNP showed a nominally significant association with plasma cortisol in the same direction as saliva cortisol.

However, the lack of genome-wide significance, low sample size, low MAF and high effect heterogeneity also cast doubt as to whether the rs6768297 association with cortisol would replicate in a completely independent sample. The SERPINA6/SERPINA1 locus identified in the plasma cortisol GWAS19 appears to be specific to plasma cortisol levels.

The results are consistent with phenotypic studies indicating that only a small proportion of cortisol variance shows a stable trait-like pattern. In three different studies Ross et al.³⁸ found that 44.4%-75.5% of total day-time cortisol output variance was under day-to-day fluctuations. Studying children through ages 9-15, Shirtcliff et al.³⁹ found that situation-specific environmental influences can explain 52% of cortisol variance (excluding circadian rhythm). The authors conclude that only 13% of the cortisol variance at a given time shows trait-like stability over the years, which coincides with the upper confidence intervals found for the heritability of acute plasma levels. These studies highlight the fact that cortisol secretion and metabolism is a highly dynamic process

adapting to not only short-term, but also long-term situational contexts, which results in considerable “noise” in genetic studies.

This notion is supported by the low heritability of the diurnal cortisol measurements. Reducing the within-day variation appears to be insufficient to reduce the contextual noise. This conclusion is further supported by the small effect adjusting for time-of-day had on the plasma cortisol estimates and the low heritability of awakening saliva cortisol. The latter has a precise circadian definition, though sampling can be difficult to time in a home environment. Furthermore, after excluding participants with plasma cortisol measurements after 11am and corticosteroid use, heritability estimates remained under 1%.

Interestingly, long-term associations between single cortisol measures in adulthood and psychosocial problems and adversities in childhood have been found.^{40,41} The variability might thus reflect environmental exposures, but for genetic studies more long-term profiles of cortisol may be needed. These can be measured using hair samples, which might represent more trait-like effects with less environmental influence.^{42,43} However, long-term environmental contexts spanning months or years also contribute to the cortisol variance and it is unclear yet to what extent 3 to 6 month measurements shall reduce environmental noise.

Therefore there may not be a single simplistic genomic heritability of cortisol levels. It is tempting to speculate that the heritability of other cortisol phenotypes is higher. Indeed the reliability of, for example, the total daily cortisol values (AUCg) is higher than single morning samples,^{13,14} but it represents a distinct feature of the cortisol secretion pattern. The cortisol awakening response or diurnal slopes are two other examples of characterizing diurnal changes. These may show a different balance of genetic and environmental influences than total daily values or hair cortisol. The awakening response or diurnal slopes may show higher heritability than the tested phenotypes, though, it should be noted that they show less stability than total daily output.³⁸ Another potentially interesting phenotype is cortisol reactivity to various stressors. Here again the heritability may be different and may even change depending on the stressor. Unfortunately, sample sizes for stress reactivity will likely be smaller. Future research is required to determine the SNP heritability of these alternative phenotypes and characterize potential differences between them, although this may be a challenging research field.

The very low diurnal cortisol heritability is in line with a twin-study reporting no genetic effects for day-time profiles.¹⁸ The same study found a non-significant heritability of 26% for awakening cortisol, which is compatible with the non-significant point estimate of 9% SNP heritability in the GREML analysis. Further, the observed 0% to 6% SNP heritability for (mostly morning) plasma and saliva levels (LD score regression) are similar to the 0% and 14% twin heritabilities reported for saliva and plasma morning

levels.^{15,18} However, they show a substantial difference to twin studies finding a 45% heritability of acute plasma levels.^{16,17}

SNP heritability is expected to be lower than twin heritability, since this estimate does not include the effects of rare, structural and X-linked variants, which are captured in twin studies. Gene-gene and gene-environment interactions can also substantially increase standard twin heritability estimates.⁴⁴ Alternatively, 45% twin heritability of acute cortisol measurements might be an overestimation, which would be consistent with the fact that the twin studies are highly inconsistent.

The LD score regression and GREML analysis of plasma cortisol in the CORNET and Rotterdam Study samples had good power to detect modest heritability. The negative findings in addition to the convergent evidence from the smaller saliva cortisol samples suggest that acute cortisol measures have low SNP heritability. However, the evidence is less clear for day-time profiles. These were only available in a small sample and have very wide confidence intervals, thus firm conclusions cannot be made. Another limitation is that the CORNET and Rotterdam Study data have an overlap in participants of approximately 20%. The samples were thus not completely independent. However, considering that the majority of the observations did not overlap and the measurements were taken at different times and assessed in different laboratories, the data nevertheless support robustness of the largely negative results.

The findings suggest that common autosomal SNPs are poor predictors of acute cortisol levels. However, predictive power is not equal to importance. Crucial cortisol regulating loci are highly conserved: mammals and fish have a similar stress physiology. Among others, corticotrophin-releasing hormone genes are orthogonal with substantial overlap in amino acid identity.⁴⁵ This highlights the importance of cortisol related genes, but also suggests that natural selection restricts the amount of variation and in turn effect sizes and predictive power. This may suggest, that if SNPs are identified despite the low SNP heritability, such as SNPs of the SERPINA6/SERPINA1 locus in the plasma cortisol GWAS, they are all the more important.

Unfortunately, it follows from the presented results, that detecting these SNPs will be difficult. Since most SNPs are expected to have a relatively low predictive contribution compared to the environment and stochastic factors, very large sample sizes are probably required to discover further loci. Given the apparent importance of cortisol genetics, GWAS seems nevertheless a worthwhile endeavor to uncover further cortisol related biological pathways.

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SUPPLEMENTAL TABLES

Table S1: Sample characteristics for CORNET cohorts participating in plasma cortisol GWAS (from Bolton et al. (2014)) and saliva cortisol GWAS.

Study	n	Men (%)	Age in years		Cortisol (nmol/l)		Sampling clock time
			M (SD)	Range	M (SD)	Range	Hr
Plasma Cortisol							
ORCADES	886	45	53.5(15.7)	17-97	765 (315)	Nov-41	0830-1030
CROATIA-Korcula	898	36	56.2 (13.9)	18-98	698 (207)	59-815	0800-0900
CROATIA-Split	496	43	45.0 (14.7)	18-85	979 (404)	94-2831	0730-0900
CROATIA-Vis	892	44	56.4 (15.5)	18-93	622 (230)	64-1820	0730-0900
RS1	2945	45	71.9 (7.0)	61-105	305 (94)	5-679	0800-1100
HBCS1934-44	451	36	60.61 (2.80)	56-67	393 (120)	125-990	0750-1055
NFBC1966	1192	0	31(0)	n/a	380 (160)	40-2370	0800-1100
ALSPAC	1567	50	15.43 (0.26)	14-17	486 (174)	58-1683	0800-1057
InChianti	1210	45	68.3 (15.6)	21-102	375 (135)	19-1291	Before 0900
PIVUS	919	50	70.2 (0.17)	69-72	386 (125)	31-930	0800-1000
PREVEND	1151	51	49.4 (13.0)	28-75	442 (201)	20-1734	0800-1100
Saliva Cortisol							
B58C-T1DGC	1762	49	45.3 (0.34)	45-46	21.1 (10.4)	3.7-61.6	0500-1100
B58C-WTCCC	1052	49	44.9 (0.35)	45- 46	21.0 (9.8)	3.7-60.8	0500-1100
NESDA	1220	33	43.5 (12.5)	18-65	17.3 (7.98)	2.0-66.6	0250-1100
NTR	162	29	29.4 (12.4)	14-75	15.8 (7.4)	2.0-46.0	0800-1100
RS1	1767	44	75.0 (5.8)	65-98	14.6 (8.5)	0.0-60.1	0248-1100
RS3	1119	43	55.3 (4.8)	46-69	14.8 (8.7)	0.7-72.0	0500-1100
MESA	166	50	67.5 (10.3)	49-90	16.2 (9.3)	2.6-89.8	Before 1100
TRAILS	455	47	16.1 (0.6)	14-17	10.9 (6.5)	1.0-112.0	0300-1100

Reference: Bolton, J.L., Hayward, C., Direk, N., Lewis, J.G., Hammond, G.L., Hill, L.A., Anderson, A., Huffman, J., Wilson, J.F., Campbell, H., Rudan, I., Wright, A., Hastie, N., Wild, S.H., Velders, F.P., Hofman, A., Uitterlin-den, A.G., Lahti, J., Räikkönen, K., Kajantie, E., Widen, E., Palotie, A., Eriksson, J.G., Kaakinen, M., Järvelin, M.-R., Timpson, N.J., Davey Smith, G., Ring, S.M., Evans, D.M., St Pourcain, B., Tanaka, T., Milaneschi, Y., Bandinelli, S., Ferrucci, L., van der Harst, P., Rosmalen, J.G.M., Bakker, S.J.L., Verweij, N., Dullaart, R.P.F., Mahajan, A., Lindgren, C.M., Morris, A., Lind, L., Ingelsson, E., Anderson, L.N., Pennell, C.E., Lye, S.J., Matthews, S.G., Eriksson, J., Mellstrom, D., Ohlsson, C., Price, J.F., Strachan, M.W.J., Reynolds, R.M., Tiemeier, H., Walker, B.R., 2014. Genome wide association identifies common variants at the SERPINA6/SERPINA1 locus influencing plasma cortisol and corticosteroid binding globulin. *PLoS Genet.* 10, e1004474. doi:10.1371/journal.pgen.1004474

Table S2: Morning Saliva GWAS: genotyping methods per cohort

Study	N	Genotyping Platform	Calling Algorithm	Reference Panel
B58C-T1DGC	1762	III 550K	Illuminus	HapMap release 21 CEU (build 35)
B58C-WTCCC	1052	Aff 500K	Chiamo	HapMap release 21 CEU (build 35)
NESDA	1220	Perlegen 600K	Birdseed	HapMap release 22 CEU (build 36)
NTR	162	Perlegen 600K	Beadstudio	HapMap release 22 CEU (build 36)
RS1	1767	Illumina Human Map 550K	Beadstudio	HapMap release 22 CEU (build 36)
RS3	1119	Illumina Human Map 610K	Beadstudio	HapMap release 22 CEU (build 36)
MESA	166	Aff 6.0	Birdseed	HapMap release 24 CEU (build 36)
TRAILS	455	Illumina BeadStation 500	Beadstudio	HapMap release 22 CEU (build 36)

Table S3: Quality Control (QC) applied to SNPs before LD score regression

QC step	Plasma	Saliva
Original SNP number	2,660,191	2,472,180
Overlap well-imputed SNP List	- 1,517,683	- 1,398,157
Missing Values	0	0
MAF \leq 0.01	0	0
Out-of-bounds p-values	0	0
Strand-ambiguity	-7	-4
Duplicated SNPs	0	0
Low sample size	-113,994	-122,663
Mismatched alleles	-29	-23
Missing LD scores	-151	-24
Final SNP number	1,028,327	951,308

