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TITLE PAGE

The Low Single Nucleotide Polymorphism Heritability of Plasma and Saliva Cortisol Levels

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Highlights

- The SNP heritability of plasma cortisol was below 10% with all analysis methods and in all samples.
- The SNP heritability of the awakening and the total diurnal cortisol in saliva was also low.
- Substantial environmental and situational factors must largely explain acute cortisol measures.
- The detection of genetic predictors of cortisol in blood or saliva will require large sample sizes.

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.

KEYWORDS

Cortisol; Genetics; Heritability; GWAS; Single Nucleotide Polymorphism

1 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 exercise (West and Phillips, 2012) or injury (Barton et al., 1987), and psychological stressors such as public speaking and demanding cognitive tasks (Kudielka et al., 2009). 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 (Adam, 2006). Additionally, cortisol is secreted rhythmically resulting in a pulsatile ultradian rhythm (Young et al., 2004). 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 (Gozansky et al., 2005; Restituto et al., 2008) and have been associated with various traits and states: BMI (Ruttle et al., 2013), cardiovascular risk factors including hyperglycaemia (Walker, 2007), psychiatric disorders, such as post-traumatic stress disorder, schizophrenia or bipolar disorder (Girshkin et al., 2014; Yehuda and Seckl, 2011) and treatment response to depression (Fischer et al., 2016). 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 (Elder et al., 2016; Golden et al., 2011).

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%) (Froehlich et al., 2000; Inglis et al., 1999; Meikle et al., 1988). Wüst, Federenko, Hellhammer, & Kirschbaum (2000) 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 familiar 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 (Bolton et al., 2014). 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 predisposition 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) (Yang, Lee, Goddard, & Visscher, 2011) in the Rotterdam Study as well as LD score regression in the CORNET GWAS results.

2 Methods

2.1 Rotterdam Study

2.1.1 Participants

The Rotterdam Study is a population-based cohort investigating chronic disease and their risk factors in elderly, see Hofman et al. (2015) 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.

2.1.2 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 $^{2}H_{3}$-cortisol as internal standard. Chromatographic separation was performed on a Waters (Milford, MA, USA) Acquity UPLC HSS T3 1.8µm 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 (Dekker et al., 2008). 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 mutidimensional 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 (The 1000 Genomes Project Consortium, 2015). SNPs included in imputation met the thresholds minor allele frequency>=1%, Hardy-Weinberg equilibrium p >10E-06, and a SNP call rate >=98.0%.

2.1.3 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 (Yang et al., 2011). 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. (2014). The plasma cortisol GREML analyses were well powered to detect 16% heritability (power=80% at α=0.05 and 2E-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. (R Core Team, 2015) 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 (Bates et al., 2014).

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.

2.2 CORNET Consortium Plasma and Saliva Cortisol GWAS

Detailed description of the CORNET GWAS on plasma cortisol can be found in Bolton et al. (2014). 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)(Power et al., 2006); the Netherlands Study of Depression and Anxiety (N=1220) (Penninx et al., 2008); the Netherlands Twin Register (N=162) (Boomsma et al., 2006), the Rotterdam Study I (N=1767); the Rotterdam Study III (N=1119); the Multi-Ethnic Study of Atherosclerosis (N=166) (Bild et al., 2002), and the Tracking Adolescents' Individual Lives Survey (N=455) (Huisman et al., 2008). 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 performed 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 (Willer et al., 2010). 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.(Turner, 2014)

2.2.1 LD Score Regression

LD Score regression exploits the relationship between SNP-Phenotype association strengths and linkage disequilibrium (LD) patterns (Bulik-Sullivan et al., 2015). 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 1.0.0 (Bulik-Sullivan et al., 2015) 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 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.

3 Results

3.1 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.

3.1.1 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 ($n_{cohorts}$= 11, $n_{participants}$=12,597). We applied LD score regression to estimate SNP heritability of plasma cortisol across multiple cohorts using the summary results of a GWAS meta-analysis. The variance explained for this larger sample was also low with 6% [95%CI: 0-13%].

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

3.2 Morning Plasma and Saliva Cortisol GWAS

The CORNET GWAS meta-analysis of plasma cortisol, which was previously published (Bolton et al., 2014), identified 4 SNPs in the SERPINA6/SERPINA1 locus, namely rs12589136, rs2749527, rs2749529 and rs11621961.

However, no SNP reached genome-wide significance ($p < 5*10^{-8}$) in the GWAS for awakening saliva cortisol. Table S4 shows results of the top 1000 associated SNPs and Figure 2 displays a Manhattan plot. Two loci showed suggestive associations ($p < 5*10^{-7}$). The T allele of rs1170109 (chr13:42779694) was associated with a 0.12 SD increase in cortisol levels (SE=0.02, $p=3.95*10^{-7}$, 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 plot (Pruim et al., 2011)). The locus was not associated with plasma cortisol (β=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, $p=2.01*10^{-7}$). Furthermore, the SNP showed a nominally significant (α=0.05) association with plasma cortisol in the same direction (β=-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).

4 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)(GTEx-Portal, 2017; Lonsdale et al., 2013). 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 GWAS (Bolton et al., 2014) 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, Murphy, Adam, Chen, & Miller (2014) 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. (2012) 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 (Power et al., 2012, 2011). 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 (Noppe et al., 2015; Rippe et al., 2015). 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 (Elder et al., 2016; Golden et al., 2011), 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 (Ross et al., 2014). 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 (Wüst et al., 2000). 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 (Froehlich et al., 2000; Wüst et al., 2000). However, they show a substantial difference to twin studies finding a 45% heritability of acute plasma levels (Inglis et al., 1999; Meikle et al., 1988).

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 (Zuk et al., 2012). 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 (Huising et al., 2004). 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.

British 1958 birth cohort

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Rotterdam Study

The Rotterdam Study is supported by the Erasmus MC University Medical Center and Erasmus University Rotterdam; The Netherlands Organisation for Scientific Research (NWO); The Netherlands Organisation for Health Research and Development (ZonMw); the Research Institute for Diseases in the Elderly (RIDE); The Netherlands Genomics Initiative (NGI); the Ministry of Education, Culture and Science; the Ministry of Health, Welfare and Sports; the European Commission (DG XII); and the Municipality of Rotterdam. The contribution of inhabitants, general practitioners and pharmacists of the Ommoord district to the Rotterdam Study is gratefully acknowledged.

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This research is part of the TRacking Adolescents' Individual Lives Survey (TRAILS). Participating centers of TRAILS include various departments of the University Medical Center and University of Groningen, the University of Utrecht, the Radboud Medical Center Nijmegen, and the Parnassia Bavo group, all in the Netherlands. TRAILS has been financially supported by grants from the Netherlands Organization for Scientific Research NWO (Medical Research Council program grant GB-MW 940-38-011; ZonMW Brainpower grant 100-001-004; ZonMw Risk Behaviour and Dependence grants 60-60600-97-118; ZonMw Culture and Health grant 261-98-710; Social Sciences Council medium-sized investment grants GB-MaGW 480-01-006 and GB-MaGW 480-07-001; Social Sciences Council project grants GB-MaGW 452-04-314 and GB-MaGW 452-06-004; NWO large-sized investment grant 175.010.2003.005; NWO Longitudinal Survey and Panel Funding 481-08-013; NWO Vici 016.130.002; NWO Gravitation 024.001.003), the Dutch Ministry of Justice (WODC), the European Science Foundation (EuroSTRESS project FP-006), Biobanking and Biomolecular Resources Research Infrastructure BBMRI-NL (CP 32), the participating universities, and Accare Centre for Child and Adolescent Psychiatry. We are grateful to all individuals who participated in this research, and to everyone who worked on this project and made it possible.

DISCLOSURES

The authors report no conflict of interest. Funding sources were not involved in study design, collection, analysis, interpretation of data, in the writing of the report and in the decision to submit the article for publication.

H.T. supervised the study. H.T. and A.N. developed the study design and drafted the manuscript. A.N. performed SNP heritability analyses. N.D. performed the meta-analysis of morning saliva GWAS. B.W. supervised the plasma cortisol GWAS meta-analysis. A.C. and S.M. performed independent shadow SNP heritability analyses. H.A. computed the genetic relatedness matrix. N.D., J.B., C.H., D.S., E.P., J.S., Y.M., B.P, J.H., E.G., A.O. and P.M. performed GWAS of morning cortisol in individual cohorts. Y.R. performed cortisol collection and analysis for the Rotterdam Study. All authors revised the manuscript critically.

All authors have approved the final article.

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Figures

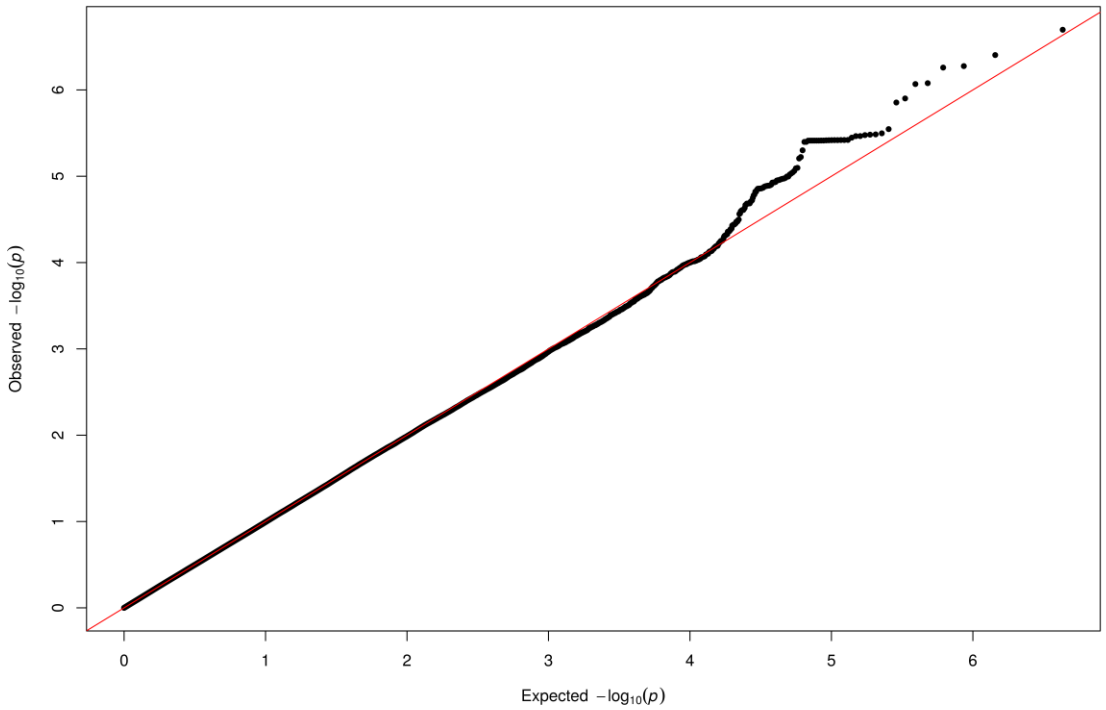


Figure 1 Quantile-quantile plot of observed -$\log_{10}$ p values vs expected -$\log_{10}$ p values assuming chance findings. Diagonal line indicates a p value distribution compatible with chance finding. Upward deviations indicate p values more significant than expected.

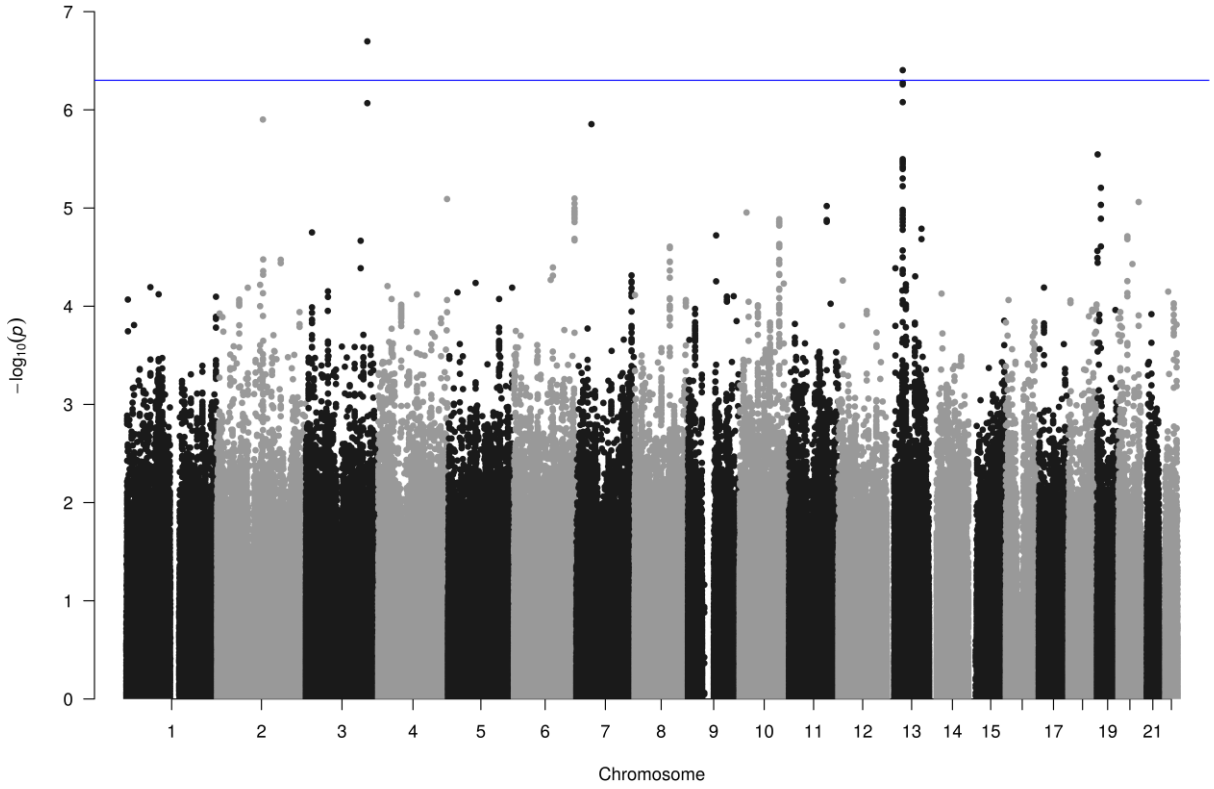
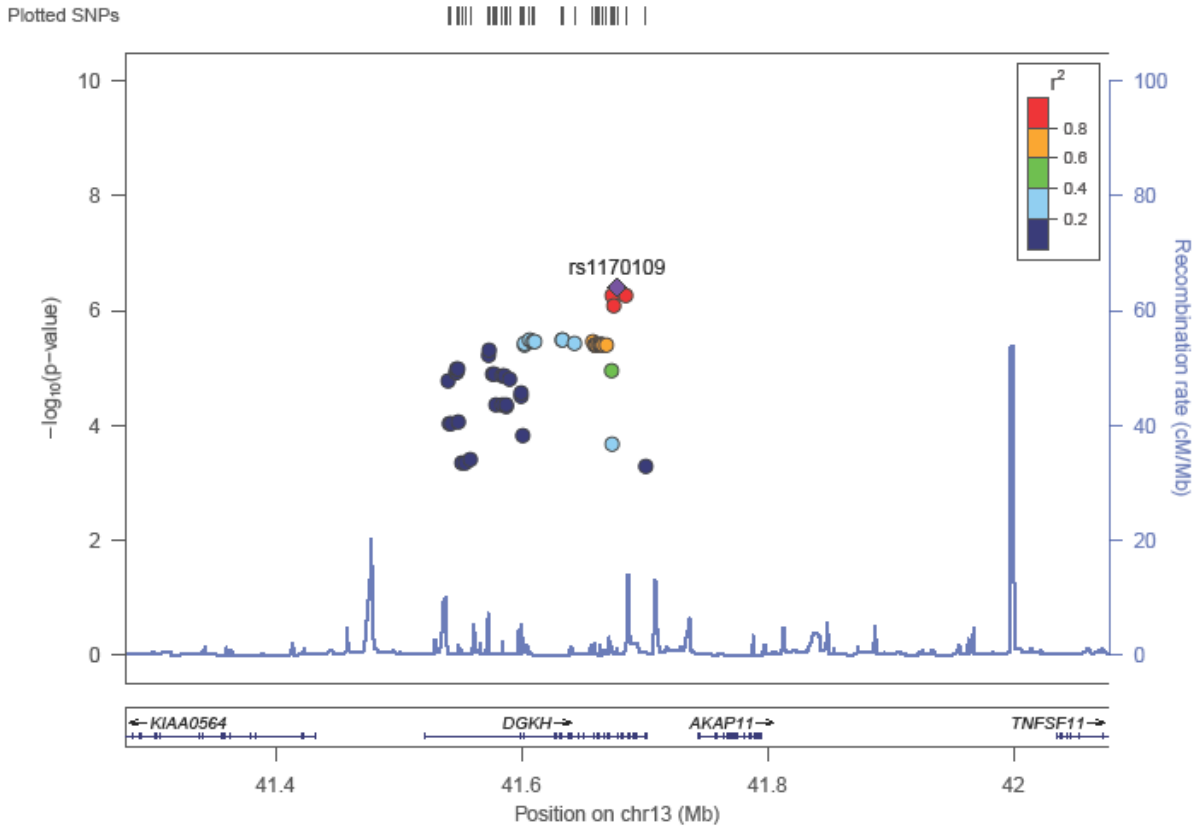


Figure 2 Manhattan plot of -$\log_{10}$ p values vs SNP position. SNPs above the horizontal line indicate suggestive findings ($p<5x10^{-7}$).



Figr

ue 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.

Tables

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%	-

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

Cortisol Phenotype	Analysis Method	Number of SNPs	n	SNP h^2	SE	p
Main Analyses:						
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	-
Sensitivity Analysis:						
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 vaiance.