CLINICAL STUDY

Individual multi-locus heterozygosity is associated with lower morning plasma cortisol concentrations

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Abstract

Objective: Stress is implicated as a risk factor for numerous illnesses in humans, putatively in part mediated by biological responses to stress, such as elevated cortisol concentrations. The theory of genetic homeostasis suggests that individual heterozygosity facilitates compensation for environmental stresses. We hypothesized that heterozygosity ameliorates the biological response to a given level of perceived stress, reflected in lower plasma cortisol concentrations.

Design: We examined the role of heterozygosity in the association between perceived psychological stress and morning cortisol concentrations in 854 individuals from the isolated island of Vis, Croatia.

Methods: Cortisol concentrations were measured in morning plasma samples. A total of 1184 autosomal microsatellite markers were genotyped and individual multi-locus heterozygosity (MLH) was calculated as the proportion of heterozygous markers. The General Health Questionnaire with 30 items (GHQ-30) was used to assess the degree of psychological distress.

Results: Mean MLH was $34.85 \pm 0.45\%$ (range: 31.97–36.22%). Psychological distress (GHQ Likert score > 31) was more prevalent in women (37 vs 18% in men, $P < 0.0001$), in less educated people ($\beta = -0.35$ per year in school, $P < 0.001$) and in lower socio-economic classes ($\beta = -3.59$, $P < 0.0001$). Cortisol concentrations were positively associated with psychological distress ($\beta = 2.20$, $P = 0.01$). In a regression model adjusted for age, BMI, education and GHQ-30 score, MLH was independently and inversely associated with morning plasma cortisol concentrations ($P = 0.005$).

Conclusion: More heterozygous individuals, as measured by microsatellite markers, had lower morning plasma cortisol concentrations for a given level of perceived psychological stress. This may be important, as higher cortisol concentrations may increase the allostatic load and be associated with a higher risk of stress-related illness.

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Introduction

Stress has been implicated as a risk factor for numerous illnesses and for mortality in humans (1, 2). However, large inter-individual differences in perception of stress, response to stress and stress-induced susceptibility to disease can alter the stress-to-illness pathway (3, 4). Relevant individual characteristics include both psychological factors, such as personality and previous experiences, and somatic differences including genetic make-up (5, 6).

The biological response to stress includes activation of the hypothalamic–pituitary–adrenal axis, which regulates the release of cortisol. Elevated cortisol

concentrations contribute to adaptive changes in immune, cardiovascular and metabolic systems to maintain homeostasis, a process called allostasis. However, sustained elevation of cortisol concentrations (as exaggerated in Cushing's syndrome) results in maladaptive changes, representing 'allostatic load'.

In 1954, Lerner (7) introduced the idea of genetic homeostasis, hypothesizing that individual heterozygosity is important because it facilitates compensation for environmental stresses and enhances an individual's ability to maintain homeostasis in changing environments. The advantages of increased locus and multi-locus heterozygosity (MLH) for disease resistance, longevity and fitness, especially when the organism is

challenged, have been described for several species including humans (8, 9, 10). However, the underlying mechanism for this 'heterozygosity–fitness correlation' is not clear, and the reported associations with infectious disease susceptibility and common complex disease risk factors are usually weak, perhaps due to the fact that most human populations are outbred with nearly uniform and high heterozygosity of individuals (11, 12, 13, 14).

A closely related and more frequently investigated research theme is inbreeding, which leads to decreased heterozygosity in offspring. The phrase 'inbreeding depression' reflects the negative effects of inbreeding and the general inferiority of the inbred offspring. Besides the well-characterized role in monogenic conditions, negative effects of inbreeding and decreased heterozygosity on fitness in humans have also been described for complex traits and diseases (14, 15, 16). Because its detrimental consequences are well known among laymen and professionals, inbreeding is discouraged in most human societies and also in agricultural and farming practices. Interestingly, it has been suggested that rural-to-urban migration and progressive globalization in the past century have led to outbreeding and consequentially to an increase in individual heterozygosity, which may have a beneficial effect on a range of human traits and could in part account for improved health and longevity (9, 15, 16, 17, 18).

We aimed to investigate the role of heterozygosity in the individual biological response to perceived psychological stress. We did so by examining the associations among psychological stress, morning cortisol concentrations and heterozygosity.

Subjects and methods

The Croatia–Vis study included 924 unselected individuals from the island of Vis, Croatia, aged 18–93 years, who were phenotyped for over 50 disease-related quantitative traits (9, 15, 19). The study received appropriate ethical committee approval, and all participants gave written informed consent. A custom-made questionnaire was used to collect information on age, socio-economic status (self-reported, estimated in comparison with that of others as 'much worse', 'a bit worse', 'same', 'a bit better' or 'much better'), education (number of completed years of education), medication, chronic illness and ancestry. Blood samples were collected between 0830 and 0930 h in the research centre after an overnight fast and were used for genetic and biochemical analyses. Individual data on medication were screened to identify individuals on corticosteroid treatment or hormone replacement therapy.

Twenty-two participants were taking corticosteroid medication and were excluded from the study. Genetic data did not pass the quality-control procedures for

further nine participants and cortisol concentration values were not available for a further 32 participants. Finally, we also excluded female participants on hormone replacement therapy ($n=7$), yielding a final sample of 854 participants who were included in the analysis.

We used the General Health Questionnaire with 30 items (GHQ-30), a screening instrument for minor psychological disorders, to assess the degree of psychological distress. It was administered by a survey team with many years of experience. The Likert method was used for scoring GHQ-30, where answers 'better than usual', 'like usual', 'worse than usual' and 'much worse than usual' are scored with 0, 1, 2 and 3 points respectively. A GHQ-30 score above 31 is considered suggestive of psychological distress when applied to a general population (20, 21). We considered scores > 2 s.d. above the mean in our study population (51 points) as indicative of severe psychological distress.

Plasma cortisol concentrations were measured using RIA (MP Biomedicals, Cambridge, UK) with an intra-assay coefficient of variation (CV) of 5.1–7.0%, an inter-assay CV of 6.0–7.9%, and a lower limit of detection of 40 nmol/l. In regression analyses, cortisol concentrations were corrected for relatedness, because our sample consisted of many related individuals, violating the independence of observation assumption that is required for the majority of statistical tests due to shared genetic factors. The GenABEL package for R allows an easy adjustment for relatedness by estimating pairwise kinship coefficients from the genome-wide data.

DNA extraction and genotyping

DNA was extracted from EDTA whole-blood specimens using the salting out procedure, stored at the MRC Human Genetics Unit (Edinburgh, UK), and genotyped at the Center for Medical Genetics, Marshfield Medical Research Foundation (USA). DNA samples were analyzed with 1184 autosomal markers comprising the microsatellite markers taken from the Marshfield Screening Set and indel markers (detailed at <http://research.marshfieldclinic.org/genetics>).

Microsatellite markers were excluded from the analysis if genotyping failed in more than 10% of all the samples and if the locus was homozygous for all the participants. We also excluded individuals for whom fewer than 750 markers were successfully genotyped, because genotyping failure is more common for heterozygous markers (22).

MLH and standardized MLH

Data on all the microsatellite markers that passed quality control were used to calculate MLH for each individual (MLH_i), that is, the proportion of heterozygous microsatellite loci among the ones that have been successfully genotyped:

$$MLH(i) = \frac{N_{\text{heterozygous}}(i)}{N_{\text{genotyped}}(i)}$$

Standardized MLH (SMLH) was calculated as MLH_i divided by the average MLH of all other n individuals, restricted to the set of loci successfully typed in the individual i .

$$SMLH(i) = \frac{MLH(i)}{\sum_{j=1}^n \frac{MLH(j)}{n}}$$

SMLH ranged from 0.9 to 1.1, with mean $\pm$ s.d. = 1.00 ± 0.03 . Calculations were performed using the Rhh application for R (available at <http://cran.r-project.org/web/packages/Rhh/index.html>).

Statistical analysis

Statistical analysis was performed using R (<http://www.r-project.org/>). The χ^2 test was used to analyse categorical variables. A multivariate regression model was constructed and morning cortisol concentration (as a continuous variable) corrected for relatedness was used as the outcome. Predictor variables used in the model were age, gender, BMI, GHQ-30 score and SMLH. To enable comparison between β -coefficients, variables were normalized to standard normal distribution and expressed as z-scores.

Results

A total of 854 participants (485 (57%) females) were included, 499 from the Komiza village and 355 from the town of Vis. The mean age of the participants was 56.56 ± 15.67 years. The median duration of education was 11 years (interquartile range (IQR): 8–12 years). The duration of education was inversely associated with age ($P < 0.0001$) and was significantly longer in males ($P < 0.0001$).

The median GHQ-30 (Likert) score was 26 (IQR: 20–33). A total of 245 participants (179 (73%) females) were categorized as suffering from psychological distress (GHQ-30 > 31) and 35 participants (25 (71%) females) had severe psychological distress (GHQ-30 > 51). In a multivariate regression analysis, we found that the GHQ-30 score was significantly associated with gender (prevalence of psychological distress was higher in females (36.9%) than in males (17.9%), $P < 0.0001$), age (β -coefficient = 0.13, $P < 0.001$), duration of education (among those with 8 or less years of full-time education, 137 individuals (40%) were distressed, in contrast to 11 (17%) among those with 16 or more years of schooling, $P < 0.001$) and socio-economic status (prevalence of psychological distress in five categories of worst-to-best socio-economic status: 70, 43, 27, 18 and 17%, $P < 0.0001$).

Mean morning plasma cortisol concentration was 619.62 ± 227.05 nmol/l. There was no statistically significant difference in cortisol concentrations between males (617.43 ± 199.86 nmol/l) and females (621.28 ± 245.92 nmol/l, $P = 0.1$), but age ($P = 0.003$) and BMI ($P < 0.001$) were inversely associated with morning cortisol concentrations.

Mean MLH (including markers that passed quality control and were not homozygous in all individuals) was $34.85 \pm 0.45\%$, range 31.97–36.22% (Fig. 1). SMLH (mean = 1.00 and s.d. = 0.03) was used in the regression analysis.

Descriptive data according to cortisol tertiles are given in Table 1. Standardized MLH was lowest in those with the highest cortisol concentrations in one-way ANOVA ($P = 0.035$) and when adjusted for age and sex ($P = 0.015$). An association between higher morning cortisol concentrations (adjusted for relatedness) and lower SMLH ($P = 0.005$) was also found in a regression model adjusted for age, GHQ-30 score and BMI (Table 2). The association remained largely unchanged after the addition of adjustment variables sex and socio-economic status to the model.

Discussion

In this study, we have shown that heterozygosity, as measured by microsatellite markers, is significantly and inversely associated with morning plasma cortisol concentrations. The association remained statistically significant after adjustment for perceived psychological distress and other measured potential confounders. This raises the possibility that the variation in the concentration of plasma cortisol, including its response to

Figure 1 Histogram of multi-locus heterozygosity in the study population.

Table 1 Characteristics of the participants by tertile of morning plasma cortisol concentrations. Data are number of participants (%) or mean $\pm$ S.D. values.

	Cortisol				<i>P</i> ^a (univariate)
	All	<508 nmol/l	508–689 nmol/l	≥ 690 nmol/l	
Cortisol (nmol/l)	619.62 $\pm$ 227.05	400.36 $\pm$ 82.49	593.12 $\pm$ 51.16	865.94 $\pm$ 188.19	
<i>n</i> (% female)	854	286 (61.5)	283 (52.7)	285 (56.1)	0.10 ^b
Age (years)	56.6 $\pm$ 15.7	57.4 $\pm$ 14.7	58.2 $\pm$ 16.0	54.2 $\pm$ 16.1	0.003
GHQ-30 (Likert points)	28.1 $\pm$ 11.3	27.7 $\pm$ 10.7	27.3 $\pm$ 11.5	29.2 $\pm$ 11.5	0.014
SMLH	0.9995 $\pm$ 0.0268	0.9999 $\pm$ 0.0288	1.0009 $\pm$ 0.0251	0.9977 $\pm$ 0.0263	0.035
BMI	27.3 $\pm$ 4.2	27.7 $\pm$ 4.1	27.6 $\pm$ 4.2	26.5 $\pm$ 4.3	<0.001
Education duration (years)	10.0 $\pm$ 3.5	10.0 $\pm$ 3.5	10.0 $\pm$ 3.5	9.9 $\pm$ 3.6	
<9 years (<i>n</i>)	348	116	111	121	
9–12 years (<i>n</i>)	364	124	124	116	
>12 years (<i>n</i>)	137	45	47	45	0.93 ^b
Unknown	5	1	1	3	
Socio-economic status					
Much worse than that of others	30	13	8	9	
A bit worse than that of others	130	46	46	38	
Like that of others	500	160	168	172	
A bit better than that of others	171	54	57	60	
Much better than that of others	19	10	3	6	0.53 ^b
Unknown	4	3	1	0	

GHQ-30, Generalized Health Questionnaire with 30 items; SMLH score, standardized multi-locus heterozygosity score.

^a*P* value for the association from the one-way ANOVA (cortisol concentration analyzed as a continuous variable).

^b χ^2 test *P* value for categorical variables (cortisol concentration analyzed by tertiles).

stressors and its contribution to allostatic load, is a mediator for the protective effect of heterozygosity on health.

The effect size of heterozygosity on cortisol concentrations was quite small (explaining 0.9% of the variance), but comparable to the effect of psychological distress found here (1.5%) and in previous studies (23, 24, 25, 26). Moreover, for other biological variables, the percentage of variance explained by heterozygosity was typically in the range from 0.07 to 3.3% and $\sim 1\%$ in studies with microsatellite markers (as has been used here) (27, 28, 29). Also, given the marked circadian variation in plasma cortisol concentration, which peaks on waking from sleep, morning cortisol values are highly variable due to variations in the time of waking, stress response to venepuncture and precise time of sampling, so it is all the more remarkable that variation attributable to heterozygosity can be detected.

The characteristics of the cohort from the island of Vis may have contributed to the detection of these small effects in a modest number of participants. The GHQ-30 questionnaire has been shown to be a valid measure of acute psychological distress in the general population (21, 30), and we observed a similarly wide range of GHQ-30 scores among the study participants as in other studies. However, the GHQ score is not a 'general stress score' and other stressors may have an effect on an individual.

The distribution of morning plasma cortisol concentrations was also similar to that we observed in several other cohorts (unpublished data), and the association with GHQ-30 scores (31, 32, 33) and other covariates

including BMI (34) was as expected. However, most human populations today are outbred and individual heterozygosity is regularly found to be distributed in a narrow range of high values. Geographical isolation of the island of Vis and the limited immigration throughout its history are reflected in the pedigree structure of the study population (15, 35). A limited choice of sexual partners may have led to a higher degree of inbreeding in this isolated population, which is reflected in the varied levels of individual MLH, increasing the power of the regression analysis when heterozygosity is used as a covariate. Sharing of similar environment and lifestyle typical for island populations is also advantageous for the analysis, in that the participants are 'naturally matched' for a range of hidden influences that could confound the analysis.

Given the limited statistical power in this study, we were unable to explore whether heterozygosity contributes to the documented associations of plasma cortisol concentrations with the well-known risk factors for

Table 2 A multivariate regression analysis of morning plasma cortisol concentrations. β -Coefficients, *P* values and percentage of variance explained are presented for the investigated predictor variables. Prior to analysis, covariates were normalized to standard normal distribution to enable the comparison of β -coefficients.

	β -Coefficient	<i>P</i>	Variance explained (%)
Age	−0.56	0.001	1.1
GHQ-30	0.39	0.009	1.5
BMI	−0.5	0.001	1.3
Standardized multi-locus heterozygosity	−0.41	0.005	0.9

disease such as blood pressure and blood glucose (34). However, both in the current cohort (9) and in that of the Framingham Heart Study (11), heterozygosity was predictive of blood pressure, blood glucose, lipid profile and urate levels as well as left ventricular diameter and ventricular wall thickness.

The mechanism for the association of heterozygosity with cortisol concentrations is unknown. It is reasonable to infer a direction of causality from genetic constitution to biological variation rather than *vice versa*. Many mutations are recessive and exhibit effects only when homozygous. An individual with low heterozygosity is more likely to be homozygous for a number of deleterious recessive alleles. Recent genome-wide association studies have shown that many common complex traits in humans are influenced by DNA sequence variants at multiple genetic loci (36). It therefore seems likely that homozygosity at numerous deleterious alleles (with individually small effects) may decrease the capacity to counterbalance stressors at an 'increased cost' in the form of increased cortisol concentrations and increased allostatic load.

On the other hand, there is the advantage of heterozygotes, or 'overdominance', which occurs when the heterozygote genotype has a higher relative fitness than either the homozygote mutant or the normal homozygote (37, 38). An example is that of sickle-cell anaemia in regions with prevalent malaria: homozygotes for mutant S-allele often die prematurely from sickle-cell disease and individuals with two normal alleles are susceptible to severe malaria, often with lethal outcomes, but heterozygous individuals are resistant to severe malaria and suffer none of the symptoms or mild symptoms of sickle-cell disease (39). By definition, less heterozygous individuals will have a smaller proportion of heterozygous loci, which will also include loci where the advantage of heterozygotes occurs. It seems likely that heterozygosity at some of the many loci likely to influence plasma cortisol concentrations is advantageous in this way. Identification of these loci, even for small effects, has the potential to flag pathways and mechanisms involved in the contribution of stress and cortisol concentrations to disease development.

Potential limitations of the study include the use of microsatellite markers to measure MLH as a proxy of true genome-wide heterozygosity. We followed previous recommendations for studying heterozygosity–fitness correlations in taking SMLH as a measure of heterozygosity and in investigating a fitness-related trait (40). However, tandem repeats are not thought to influence gene function directly (and are, therefore, not influenced by selection pressure directly), so we infer that heterozygosity of other genetic variants is mechanistically important in the protective effect, but we have not investigated those here.

Another limitation is that it is unknown how much time passed between waking and blood sampling for our

participants, so not all would have been sampled at their peak cortisol concentrations (30–45 min after waking) (41) and cortisol concentrations might have been further elevated due to the stress related to the clinic visit and venepuncture. However, psychological distress has been shown to be associated with 'morning cortisol' concentrations measured in this way and not with just peak concentrations after waking (31, 32).

We conclude that in this cohort more heterozygous individuals had lower morning plasma cortisol concentrations for a given level of perceived psychological distress. This may be important as higher cortisol concentrations may increase the allostatic load and be associated with a higher risk of stress-related illness. In the future, alternative tools to SMLH, such as the analysis of 'runs of homozygosity', may be used to explore the generalizability of these findings in larger cohorts (42).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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